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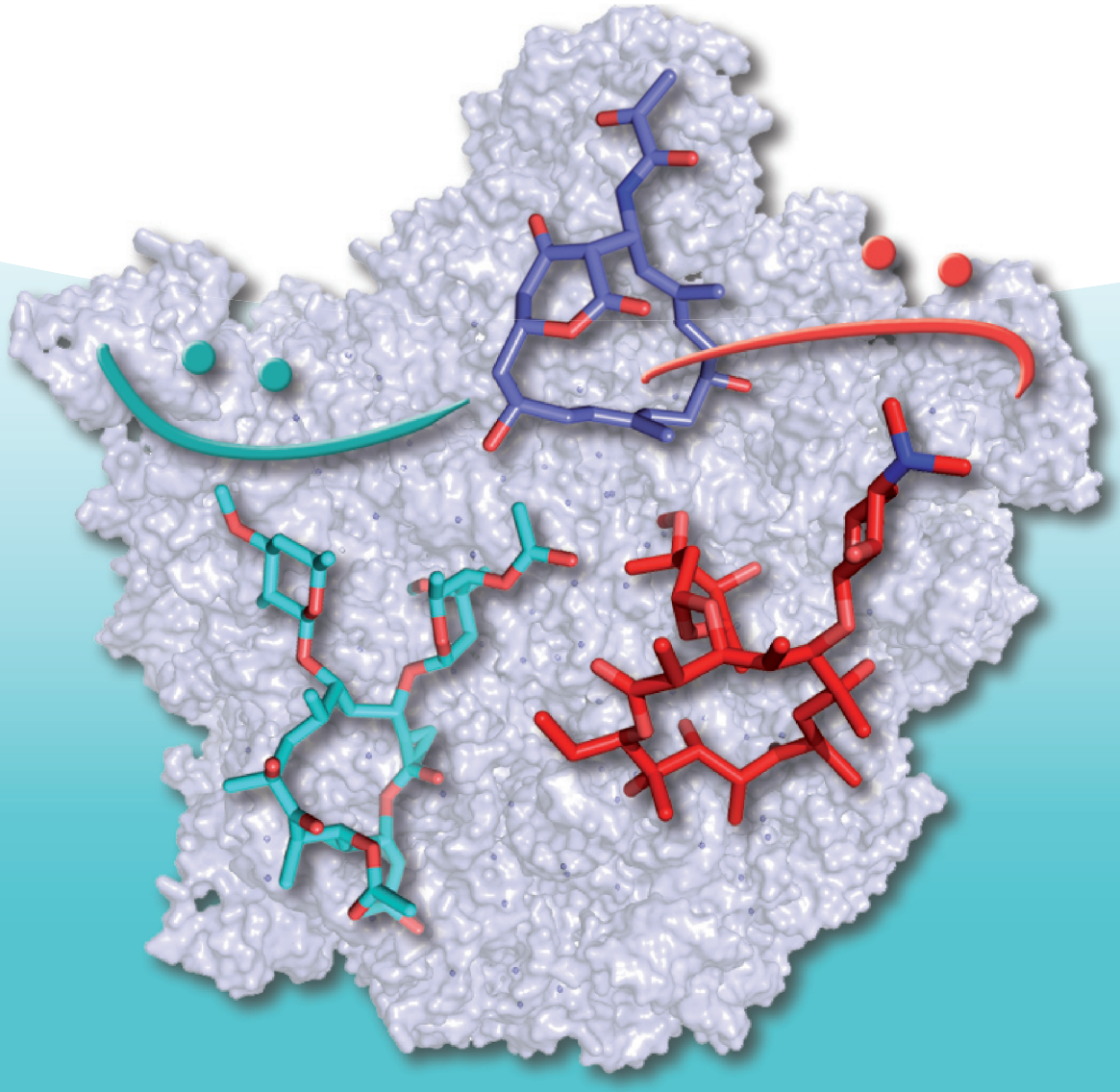
Antibiotics

Targets, Mechanisms and Resistance



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Targets, Mechanisms and Resistance

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Preface

Resistance to antibiotics has increased and is still growing so that almost every human pathogen has acquired resistance to at least one class of antimicrobials that are in clinical use. The fairly large number of fatalities caused by untreatable bacterial infections in recent years underlies the existence of an antibiotic-emergency, which renders formidable the health threat caused by infectious diseases by both conventional pathogens and emerging killer ''superbugs''.

It seems clear that the drive of big pharmaceutical companies toward research and development of anti-infectives is long gone and has rapidly brought to an end the golden era of antibiotics. Nevertheless, the world is currently experiencing an increasing demand for therapeutic means to fight and overcome infectious diseases responsible for the majority of nosocomial infections and deaths.

Aside from economic reasons, a number of frustrating and expensive strategic mistakes have undoubtedly contributed to determine the disengagement of the big pharma and the consequent present shortage of antibiotics. Several recent publications have dealt with the analysis of ''what went wrong'' in antibiotic research; despite some understandable differences in evaluating the specific significance that different factors have played in generating the present situation, there is almost unanimous consensus in identifying at least some of the causes of the past failures. In turn, the lessons learned from these mistakes now form the basis for designing new strategies for the discovery and development of new antibiotics. More specifically, great hopes have been placed on the design of miniaturized, intelligent *in vitro* or *in vivo* screening tests, some of which are directed towards identifying inhibitors of novel or underexploited targets, on the use of new generation repertoires of select natural compounds instead of large chemical libraries, and on bioinformatics, NMR- and crystallography-based technologies such as fragment-based drug discovery and structure-based rational design. Also, the rediscovery of molecules detected and subsequently neglected during the golden years of antibiotic research may prove to be an excellent starting material for the successful development of anti-infective agents.

Beyond the enormous impact that antibiotics had in safeguarding human health over the last half century, the paramount importance of these compounds in contributing to the progress of science, genetics, and molecular biology in

particular, should not be neglected. Indeed, the number of fundamental biological functions whose molecular mechanisms have been elucidated with the help of antibiotics is countless, as is the number of essential genes, such as those encoding the two subunits of gyrase, the elongation factor EF-G, and an entire cluster of ribosomal protein genes, to name a few which have been identified and initially characterized through the study of antibiotic resistance.

In light of these considerations, in addition to a few chapters that are devoted to general aspects such as a survey of the chemical classes of antibiotics and antibiotic resistance and fitness cost of resistance, most of the chapters of this book cover individual biological functions and biomolecules representing specific antibiotic targets. In this way, the reader should be able to appreciate the strict inter-relationship between biological mechanisms, on the one hand, and the nature and mechanism of inhibition of antibiotics, on the other.

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1
A Chemist's Survey of Different Antibiotic Classes

Sonia Ilaria Maffioli

1.1
Introduction

More than 20 novel classes of antibiotics were produced between 1930 and 1962. Since then, only four new classes of antibiotics were marketed. Interestingly, none of these new classes is really novel: daptomycin, approved in 2000, was discovered in the early 1980s; linezolid, approved in 2000, derives from a synthetic lead discovered in the 1970s; pleuromutilins, approved in 2007, have been widely used for about 30 years in veterinary medicine; fidaxomicin, approved in 2011, was first reported in the 1970s. This chapter reviews the main classes of antibiotics in clinical use organized by their chemical structure. For each class, the natural or synthetic origin and a description of the chemical structure are presented. The mechanism of action and spectrum of activity are only briefly indicated as they are discussed more deeply in the subsequent chapters. A short summary of the early structure–activity relationships (SARs) leading to the most known derivatives is described followed by a short overview of the most recent analogous currently under clinical development [1–3].

1.2
Aminoglycosides

Aminoglycosides (Figure 1.1) were first established as antibiotics in the 1940s and are still widely used worldwide. They are obtained by fermentation of *Streptomyces*, *Micromonospora*, and *Bacillus*; irreversibly inhibit protein synthesis by acting on the ribosome; and are especially active against gram-negative bacteria. They chemically consist of an aminocyclitol substituted with amino sugars. A classification proposed by Umezawa was based on the central structure, which can be streptamine **1**, 2-deoxystreptamine **2**, or streptidine **3**. A relevant number of natural and semisynthetic derivatives have been obtained since their discovery with the aim of bettering the toxicity issues linked to these structures, mainly oto- and nephrotoxicity, and to fight the increased resistance that mostly arises from structural modification of the

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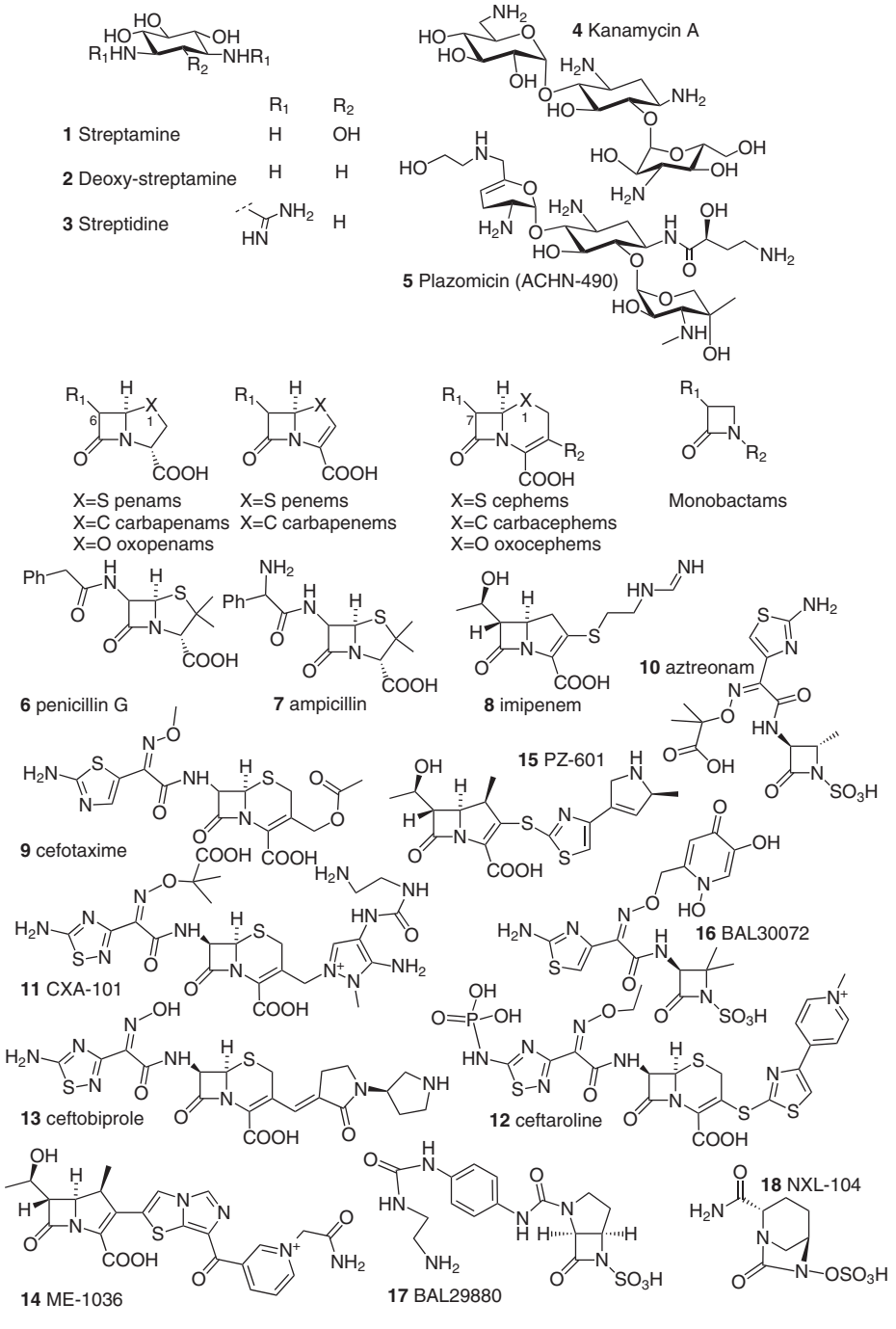


Figure 1.1 Aminoglycosides and β-lactam antibiotics.

aminoglycosides by specific enzymes expressed by resistant strains. These studies highlighted the importance of the number and position of the amino groups for the antibacterial activity. For example, the derivatization of the amino and alcoholic groups in kanamycin **4** resulted in an increased potency together with a reduced susceptibility to the inactivating enzymes that act by acetylation of 2′- and 6′-position and by phosphorylation on position 3′. Recently, interest in this class increased again owing to their spectrum of activity and the observed synergistic activity with other antibiotic classes [1]. Among the recent derivatives, plazomicin (ACHN-490) **5**, a semisynthetic derivative of sisomycin, shows significant improved activity against amikacin- and/or gentamicin-resistant strains and is currently under phase II clinical study [2, 3].

1.3 β-Lactams

β-Lactam antibiotics, discovered in the 1930s and produced by the fungus *Penicillium*, are a wide class of antibiotics, characterized by the presence of an azetidinone nucleus containing the carbonyl β-lactam, essential for the activity. Different subclasses of β-lactams can be defined depending on the chemical substitutions of the central β-lactamic core (Figure 1.1). The azetidinone can be fused with a saturated or unsaturated pentacycle or hexacycle and position 1 of this ring can be occupied by a sulfur, oxygen, or carbon atom. Thus, penicillins, including penams, carbapenams, and oxopenams, contain a saturated pentacle (see penicillin B **6** and ampicillin **7**), penems, and carbapenems contain an unsaturated pentacycle (imipenem **8**) and cephalosporins, including cephems, carbacephems, and oxacephems, contain an unsaturated hexacyle (cefotaxime **9**). Finally, the azetidinone can be alone and not fused with another ring originating monolactams or monobactams (aztreonam **10**). All β-lactams act on cell-wall biosynthesis, targeting the penicillin-binding protein (PBP) enzymes involved in the biosynthesis of the peptidoglycan. In the many decades after penicillin discovery in the 1930s, a huge number of natural, synthetic, and semisynthetic β-lactams were discovered and produced [4]. Initially, position 6 of penicillin was extensively modified to increase the stability of the β-lactam and to overcome the resistance mostly mediated by the production of a PBP with reduced affinity for β-lactams. In cephalosporins, a similar approach by modification of the side chain in position 7 gave rise to new generations of semisynthetic cephalosporins. Initially active mainly on gram-positive bacteria, newer generations have significantly greater gram-negative antimicrobial properties. In the next generations, the *N*-acyl side chain was then coupled with structurally complex heterocycles at position C-3 containing a positive charge at their terminus (Figure 1.1) [5]. The resulting chephalosporins CXA-101 **11**, ceftaroline **12**, and ceftobiprole **13** have exceptional gram-positive activity that also crosses over to some gram negatives [6]. The same type of positively charged heterocycle was also incorporated in position C-2 of the carbapenems (ME-1036 **14**). The injectable carbapenem PZ-601 **15** has shown potent activity against drug-resistant gram-positive

pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), and is currently undergoing phase II studies. Among monobactams, in which aztreonam is the only representative widely used in clinics, the newest generation incorporates a siderophore substructure to facilitate bacterial uptake (BAL-30072 **16**). Finally, combinations of a β-lactam with a β-lactamase inhibitor have been successfully used to achieve antibacterial efficacy without accelerating resistance development. Clavulanic acid was the first β-lactamase inhibitor used in combination drugs followed by sulbactam and tazobactam, and more recently BAL29880 **17**, all possessing a β-lactam chemical structure [5]. Recently, a novel bicyclic, non-β-lactam β-lactamase inhibitor (NXL104 **18**) is under clinical evaluation [7].

1.4 Linear Peptides

In this family, gramicidins, dalbaheptides, and lantibiotics are grouped. In all these molecules, the main peptidic chains remain linear and no cyclization occurs at the N- or C-terminal amino acid, yet rings can be present because of cyclization between side chains belonging to different residues. Gramicidin D is a heterogeneous mixture of six strictly related compounds, gramicidins A, B, and C obtained from *Bacillus brevis* and collectively called *gramicidin D* [8]. In contrast to gramicidin S, which is a cyclic peptide, gramicidin D contains linear pentadecapeptides with alternating L- and D-amino acids, sharing the general formula: formyl-L-*X*-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-*Y*-D-Leu-L-Trp-D-Leu-L-Trp-ethanolamine, where amino acids *X* and *Y* depend on the gramicidin molecule. *X* can be Val and iLeu, while *Y* represents an aromatic amino acid among which are Trp, Phe, and Tyr. The alternating stereochemical configuration (in the form of D and L) of the amino acids is crucial for antibiotic activity. In membranes, gramicidin adopts a β-helix three-dimensional conformation forming channels that are specific to monovalent cations, thus increasing the permeability of the bacterial cell membrane and thereby destroying the ion gradient between the cytoplasm and the extracellular environment.

1.4.1 Glycopeptides-Dalbaheptides

Dalbaheptides (Figure 1.2) are composed of seven amino acids cross-linked to generate a rigid concave shape. This configuration forms the basis of their particular mechanism of action that involves the complexation with the D-alanyl-D-alanine terminus of bacterial cell-wall components. As this mechanism of action is the distinguishing feature of these glycopeptides, the term *dalbaheptide*, from D-al(anyl-D-alanine)b(inding)a(ntibiotics) having hept(apept)ide structure, has been proposed to distinguish them within the larger and diverse groups of glycopeptide antibiotics [9]. Five of the seven amino acids forming the peptidic skeleton are common to all dalbaheptides. Vancomycin **19**, the first dalbaheptide introduced into clinical

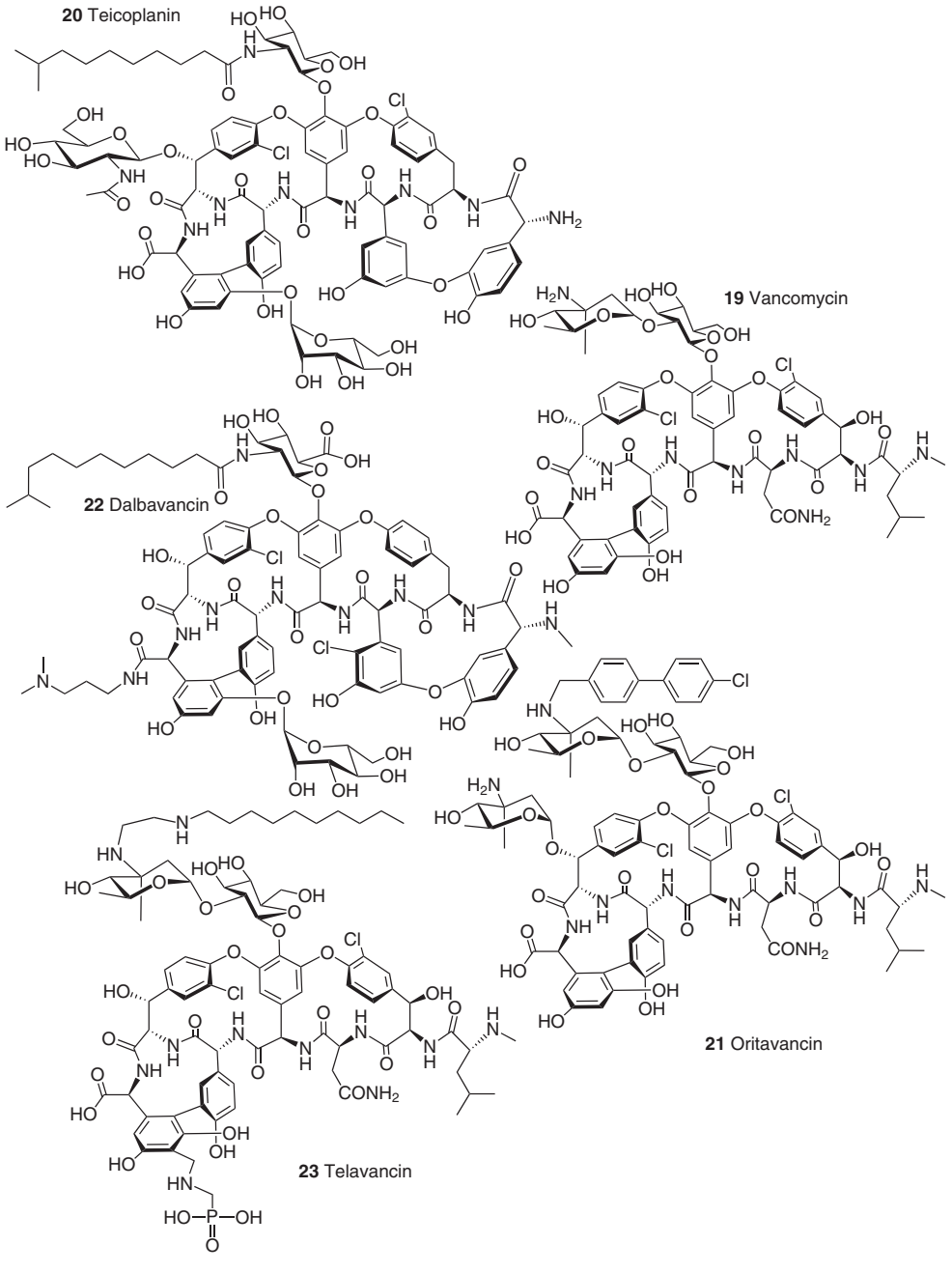


Figure 1.2 Dalbaheptide antibiotics.

practice in 1959, was isolated from *Streptomyces orientalis* (now *Amycolatopsis orientalis*) [4]. In 1988, teicoplanin **20** was also introduced. Glycopeptide antibiotics are restricted to treating gram-positive infections as they cannot penetrate the outer membrane of gram-negative bacteria. As vancomycin has been increasingly used for the treatment of a wide range of infections, second-generation glycopeptides with improved profile over vancomycin were developed. Even though recently innovative synthetic methods allowed successful total syntheses of these complex structures, fermentation followed by semisynthetic modification remains the prevalent way to explore SARs and the only practicable route to bulk production of clinical candidates. In general, the presence of specific sugars is of vital importance for dalbaheptide activity as aglycones are uniformly less active. At the same time, most efforts to change the natural heptapeptide backbones have resulted in reduced activity. Nevertheless, modification of the natural structure has led to novel, resistance-breaking dalbaheptides that contain structural elements promoting dimerization, to tight binding with the biological target, and lipophilic side chains that enhance membrane anchoring. An additional amino sugar at residue 6 and aromatic chlorine substituents promote favorable dimerization, and substitution of the free carboxylate function by basic carboxamides increases the activity against staphylococci. From these studies, three semisynthetic second-generation drugs have been advanced to clinical development. Oritavancin **21**, derived from the vancomycin-related glycopeptide chloroeremomycin, dalbavancin **22**, a derivative of the teicoplanin-related glycopeptide A40926, and telavancin **23** were approved by the Food and Drug Administration (FDA) in the United States in 2009 [10, 11].

1.4.2 Lantibiotics

Lantibiotics are small peptides (19–38 amino acids) produced mostly from strains belonging to the Firmicutes and, to a lesser extent, to the Actinobacteria, that undergo extensive posttranslational modifications to yield the active structures. The modifications common to all lantibiotics involve the dehydration of serine and threonine residues to yield 2,3-didehydroalanine (Dha) and (Z)-2,3-didehydrobutyrine (Dhb), respectively (Figure 1.3). This is followed by the stereospecific intramolecular addition of a cysteine residue onto Dha or Dhb to form a lanthionine (Lan) or methyllanthionine (MeLan) bridge, respectively. The term *lantibiotic* is, in fact, derived from Lan-containing antibiotics. Other modifications can be present on these molecules: for instance, C-terminal Cys residues may form *S*-aminovinylcysteine (AviCys) while N-terminal residues can contain 2-oxopropionyl (OPr) and 2-oxobutyryl groups (OBu). Their antimicrobial activity is limited to gram-positive bacteria; the prototype molecule is nisin **24**, discovered in the 1920s and used as a food preservative for 40 years [12]. Lantibiotics are divided into two classes according to their biogenesis: Lan formation in class I compounds requires two separate enzymes, a dehydratase and a cyclase, whereas a single enzyme carries both activities for class II lantibiotics. Although compounds from both classes exert their

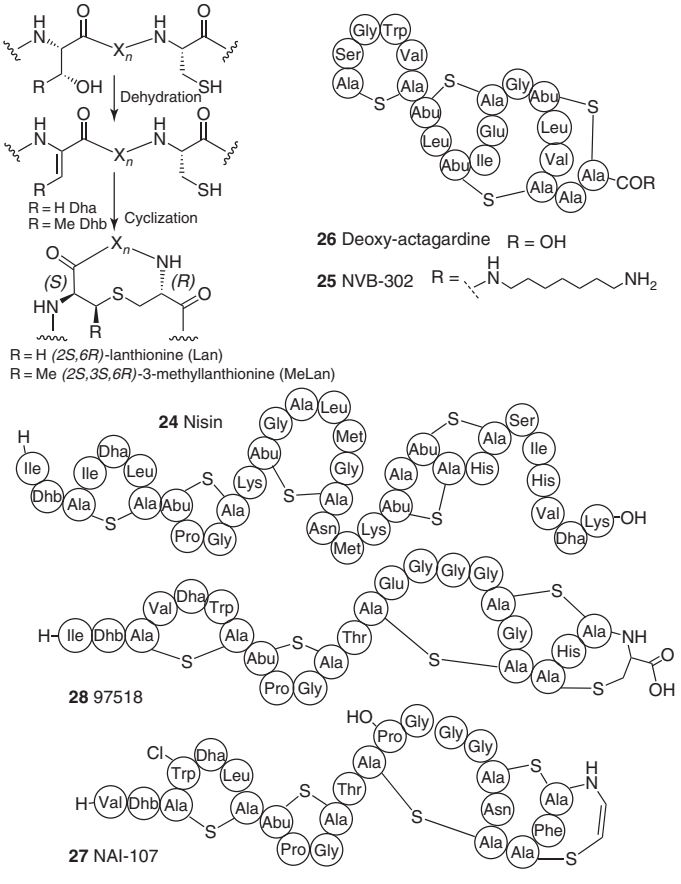


Figure 1.3 Lantibiotics.

antimicrobial activity by binding to Lipid II, thus inhibiting cell-wall biosynthesis, they do so by binding to different portions of this key peptidoglycan intermediate. Moreover, lantibiotics bind Lipid II at a site different from that affected by vancomycin and related glycopeptides, and they are active against multidrug-resistant (MDR) gram-positive pathogens and have attracted attention as potential drug candidates. The compound NVB302 **25**, a semisynthetic derivative of deoxyactagardine B **26**, is currently a developmental candidate [13]. Independently, a screening program designed to detect cell-wall-inhibiting compounds turned out to be very effective in identifying lantibiotics [14]. Among the new lantibiotics identified, the most active compound was NAI-107 **27**, containing two previously unknown modifications: a chlorinated tryptophan and a mono- or dihydroxylated proline. It is currently a developmental candidate for the treatment of nosocomial infections by gram-positive pathogens [15]. The same screening program led to the identification of additional class I lantibiotics from actinomycetes. Among them, the compound 97518 **28** is structurally related to NAI-107 but contains two carboxylic acids [16]

(the unmodified carboxy-terminal amino acid and an aspartic residue) and afforded improved derivatives by chemical modification of the acidic residues [17].

1.5 Cyclic Peptides

For simplicity, all cyclic peptides are grouped in this family (Figure 1.4 and Figure 1.5). Nevertheless, while the first described gramicidin S is a simple

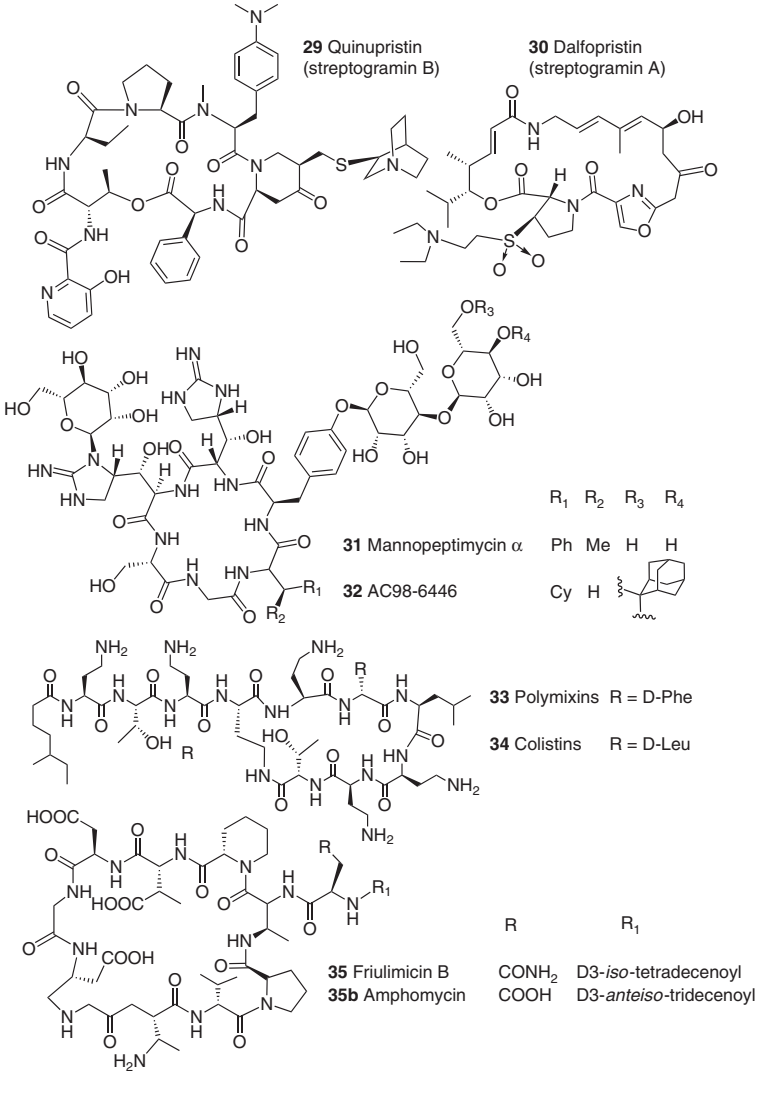


Figure 1.4 Cyclic peptide antibiotics (part I).

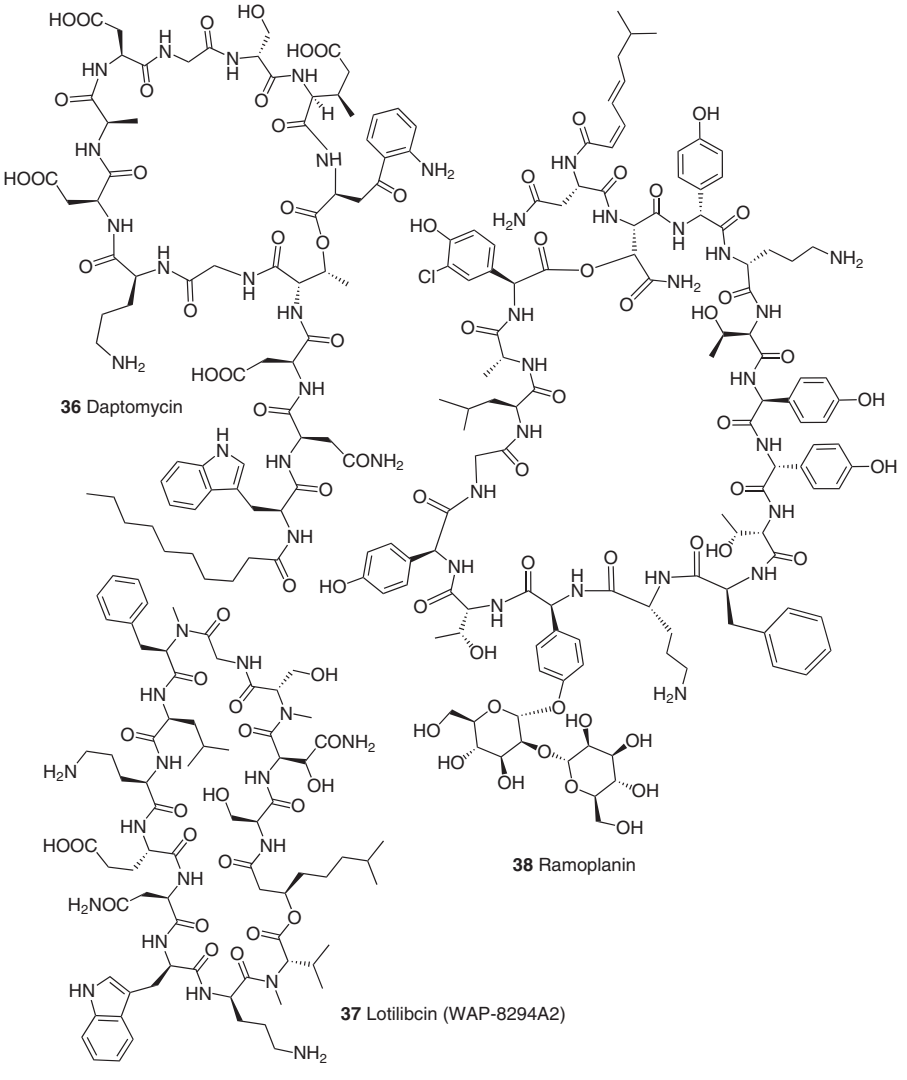


Figure 1.5 Cyclic peptide antibiotics (part II).

cyclopeptide, the later described antibiotics are more complex structures containing additional chemical groups that identify the molecules as glycosylated peptides when they contain sugar moieties (mannopeptimycin **31**), lipopeptides when they contain a lipophilic side chain (polymixin **33**, friulimicin **35**), lipodepsipeptides when apart from the lipophilic chain a lactone is present in the cycle (daptomycin **36**, lotilibcin **37**), and glycolipodepsipeptide when all these characteristics are present (Ramoplanin **38**). Gramicidin S is an antibiotic effective against some gram-positive and gram-negative bacteria as well as some fungi, which was discovered in 1942 and produced by the gram-positive bacterium *B. brevis*. Gramicidin S is

a cyclodecapeptide, constructed as two identical pentapeptides joined head to tail, formally written as cyclo(-Val-Orn-Leu-D-Phe-Pro)$_2$. Streptogramins are natural products produced by various members of the *Streptomyces* genus. This family of antibiotics consists of two subgroups, A and B, which are simultaneously produced in a ratio of roughly 70 : 30 [18]. Both subgroups inhibit protein synthesis by binding to the ribosome. Group A streptogramins are cyclic polyunsaturated macrolactones. Structural variations in type A streptogramins can arise from desaturation of the proline residue and by its substitution for alanine or cysteine residue. Examples of group A streptogramins are pristinamycin IIA (same as virginiamycin M1), madumycin II, and the semisynthetic derivative dalfopristin **29**. Group B streptogramins are cyclic hepta- or hexadepsipeptides, for example, pristinamycin IA, virginiamycin S, the semisynthetic quinupristin **30**. The invariant N-terminal threonine residue is N-acetylated with 3-hydroxypicolinic acid and forms a cyclizing ester linkage with the C-terminal carboxyl group of the peptide via its secondary hydroxyl group. Synercid (composed of a mixture of quinupristin and dalfopristin) is not orally available and is administered by intravenous routes. Efforts have therefore been made to generate new orally active streptogramins. In particular, a new oral streptogramin, designated NXL-103, has been shown to be very effective against a number of gram-positive and gram-negative organisms. Mannopeptimycins [19] are glycosylated cyclic hexapeptides that contain both stereoisomers of the unusual amino acid β-hydroxy-enduracididine. They also contain an unusual N-glycosidic bond, which links a mannose sugar to one of the β-hydroxy-enduracididine residues. They were originally isolated in the 1950s from *Streptomyces hygroscopicus* but the chemical complexity and the lack of broad-spectrum activity reduced prospects for further development. Mannopeptimycin **31** affects cell-wall biosynthesis and recently renewed interest in it has derived from its activity against MDR gram-positive pathogens. SAR data derived from the natural congeners, chemical derivatization, precursor-directed biosynthesis, and pathway engineering were employed for optimization [20]. These data demonstrated that antibacterial activity was enhanced by hydrophobic *O*-acylation of either of the two *O*-mannoses, particularly the terminal one, while it was reduced by esterification of the N-linked mannose or serine moieties. AC98-6446 **32** represents an optimized lead obtained by adamantyl ketalization of a cyclohexyl analog prepared by directed biosynthesis. Polimixins **33** and colistins **34** have a general structure consisting of a cyclic peptide with a long hydrophobic tail [21, 22]. They are produced by the gram-positive *Bacillus polymyxa* and are selectively toxic for gram-negative bacteria owing to their specificity for the lipopolysaccharide (LPS) molecule that characterizes many gram-negative outer membranes. The hydrophobic tail is important in causing membrane damage, suggesting a detergent-like mode of action. Polymixin nonapeptide, devoid of the hydrophobic tail, still binds to LPS, and causes some degree of membrane disorganization but no longer kills the bacterial cell. Polymixin B (colistin) was approved for clinical use in 1958 but its systemic toxicity, particularly nephrotoxicity, has limited its use to topical applications for the most part. Nevertheless, currently, polymyxins have been revived to treat infections due to multiply resistant gram-negative bacteria. Friulimicin B **35**

consists of a macrocyclic decapeptide core with an exocyclic asparagine acylated with a branched unsaturated lipophilic chain. Structurally, friulimicin belongs to the amphomycin family of cyclic lipopeptides, whose members differ in amino acids and fatty acid substituent. The correct structure of amphomycin (**35b**) was actually established almost 50 years after its discovery, along with friulimicin characterization. These studies revealed that the friulimicin producer *Actinoplanes friuliensis* makes macrocyclic decapeptides with an exocyclic acylated aspartic residue, identical to previously described amphomycin, tsushimycin, parvuline, and aspartocin, as well as compounds with an exocyclic asparagine, such as friulimicin [23]. Notwithstanding the structural similarity to daptomycin, amphomycin has a different mechanism of action; it has long been known to inhibit cell-wall biosynthesis and has completed phase I clinical trials. Daptomycin **36** is a cyclic lipodepsipeptide produced by *Streptomyces roseosporus* consisting of 13 amino acid cyclic peptides with a decanoyl side chain [24]. Discovered in the late 1980s, it is the first lipopeptide approved for clinical use (2003) in the treatment of gram-positive infections. Daptomycin acts on the membrane and causes rapid depolarization, resulting in a loss of membrane potential leading to inhibition of macromolecular syntheses and ultimately bacterial cell death. Its distinct mechanism of action means that it may be useful in treating infections caused by MDR bacteria. Lotilibcin (WAP-8294A2) **37** is a complex of 20 closely related components produced by a gram-negative bacterium *Lysobacter* sp. They are cyclic depsipeptides containing 12 amino acid residues and one 3-hydroxy-fatty acid residue. WAP-8294A2 was isolated as the major component, and showed a strong activity against gram-positive bacteria without posing any cross-resistance [6]. Ramoplanin **38** is a glycolipodepsipeptide antibiotic isolated from fermentation of *Actinoplanes* sp. containing 17 amino acids and a mixture of L and D amino acids as well as several nonproteinogenic side chains [25]. The first amino acid in the depsipeptide is acylated at the amino terminus with a lipid unsaturated substituent slightly different for three congeners. It is active against gram-positive aerobic and anaerobic bacteria, including vancomycin-resistant enterococci. Ramoplanin inhibits bacterial cell-wall biosynthesis by a mechanism different from those of other cell-wall synthesis and therefore does not show cross-resistance with them. Because of its potent antimicrobial activity, ramoplanin could be an effective antibiotic for treating serious gram-positive infections. However, it showed poor local tolerability upon intravenous injection and it is under development for prevention and treatment of *Clostridium difficile*-associated diarrhea, acting locally by decolonizing the gut. Semisynthetic derivatives of the natural molecules have been produced by selective removal and replacement of the original fatty acid chain with different chemical residues [26].

1.6 Thiazolylpeptides

Thiazolylpeptides are highly modified, ribosomally synthesized peptides that inhibit bacterial protein synthesis. They are characterized by a sulfur-containing

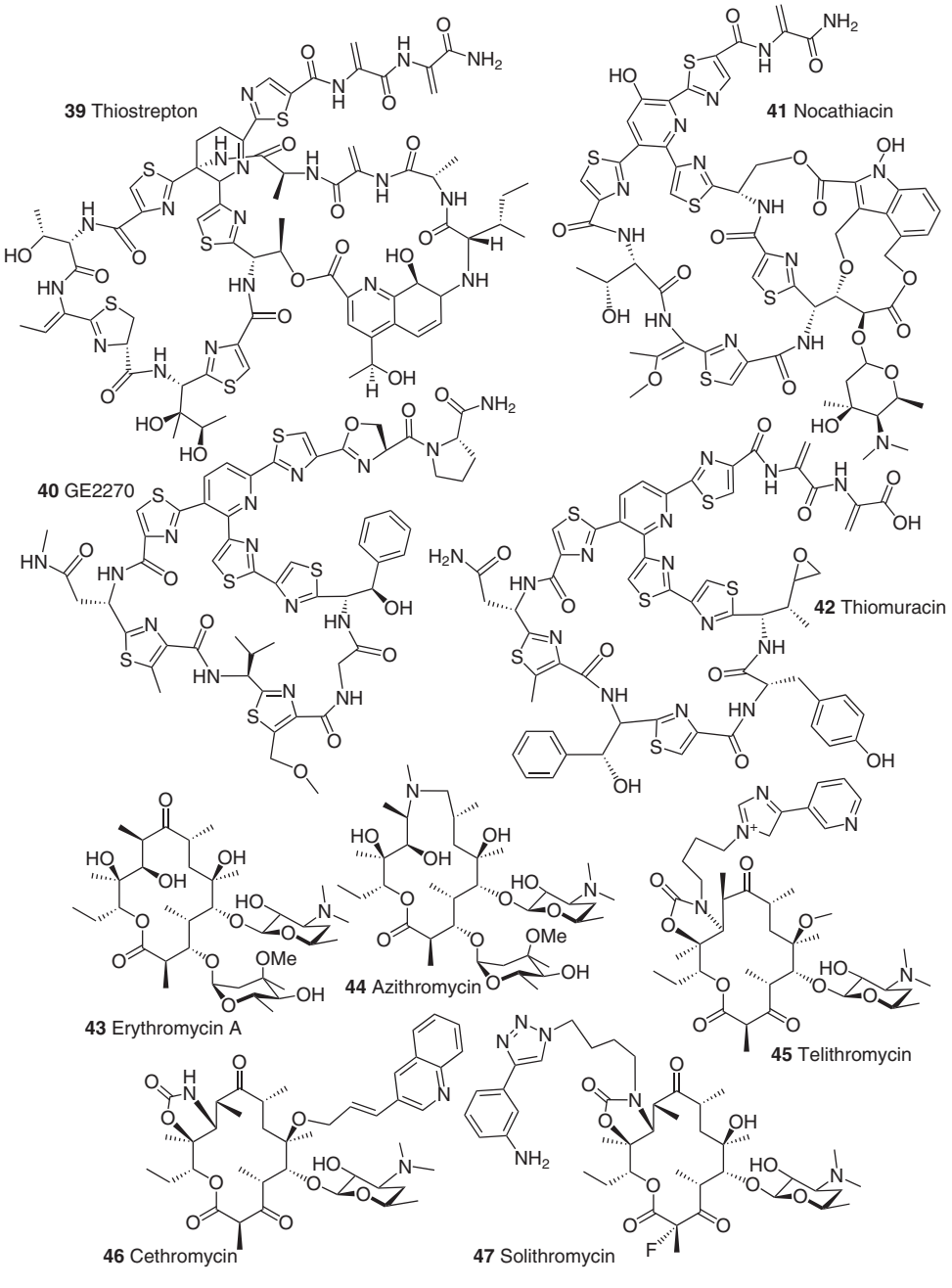


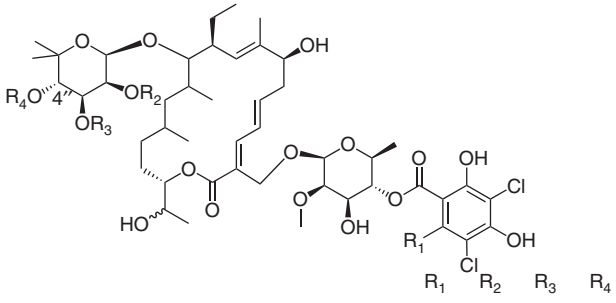
Figure 1.6 Thiazolylpeptide and macrolide antibiotics.

macrocyclic structure, which possess a tri- or tetra-substituted nitrogen-containing heterocycle core (Figure 1.6). Micrococcin was the first thiopeptide ever discovered (1948); it was produced by a *Micrococcus* sp. Other members of this class are produced by *Streptomyces* (thiostreptone **39**) and *Planobispora* sp. (GE2270 **40**). Nearly all of the thiopeptides inhibit protein synthesis; however, their cellular targets are distinct. For example, the structurally complex polycycles of nocathiacin **41** [27] and thiostrepton **39** bind to the 23S ribosomal ribonucleic acid (rRNA) component of the bacterial 50S ribosomal subunit, while GE2270 **40** and the thiomuracin **42** monocycles target the elongation factor Tu [28]. Most thiazolylpeptides show potent activity against gram-positive pathogens and this unique class of thiopeptides represents a significant and promising lead for antibiotic drug discovery, yet their poor solubility has limited clinical progress; only a derivative of GE2270 has entered clinical trials for the topical treatment of acne (NAI-Acne), in which the natural carboxy terminal is replaced by a semisynthetic amide residue [11]. Additional novel derivatives of GE2270 have recently been identified, where the natural carboxy-terminal amino acids are replaced by cycloalkylcarboxylic acid side chains by amide or urethane bond [29], and novel water-soluble derivatives of nocathiacin were also recently reported [27].

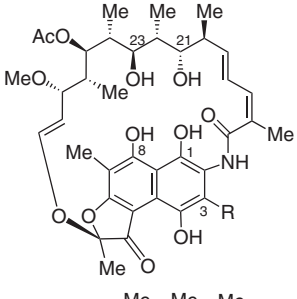
1.7 Macrolactones

1.7.1 Macrolides

Macrolides (Figure 1.6) are composed of a macrolacton, usually of 14–16 atoms, and at least 2 neutral- or amino sugars linked to the macrocycle, usually cladinose and desosoamine. Erythromycin A **43**, the prototype of this class, was first isolated from *Streptomyces erythreus* in 1952 [4]. Since their discovery, a significant number of new natural and semisynthetic derivatives have been produced. Despite the availability of total synthesis tools, semisynthesis still remains the only possibility for all marketed macrolides; nevertheless, molecular diversity was obtained in macrolides, not only by classical semisynthesis but also by combinatorial biosynthesis through modification of the polyketide biosynthetic machinery [4]. Among the most interesting semisynthetic derivatives, the azalides, obtained by Beckmann rearrangement from erythromycin A, have increased activity against gram-negative bacteria. Among them, azytromycin **44** was commercialized at the end of the 1980s. More recently, to overcome the several mechanisms involved in the resistance against this class, new macrolides named ketolides were rediscovered, in which 3-cladinose, erroneously considered for many years as a crucial structural element for antibiotic activity, is replaced with a 3-ketone substituent. Novel ketolides were demonstrated to have increased stability in acidic media and potent activity against erythromicin- and penicillin-resistant enterococci together with an enhanced pharmacokinetic profile. Among them, telithromycin **45** was the



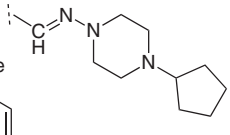
	R_1	R_2	R_3	R_4
48 Difimicin (tiacumicin B, lipiarmycin A_3, clostomicin B1)	Et	H	H	*i*-PrCO
49 Lipiarmycin B_3 (tiacumicin B_2, clostomicin C)	Et	*i*-PrCO	H	H

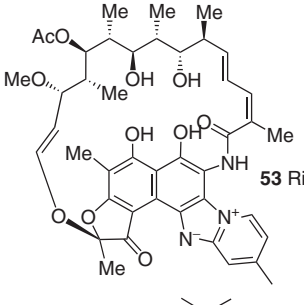


50 Rifamycin SV R = H

51 3-formyl rifamycin SV R = CHO

52 Rifapentin R =

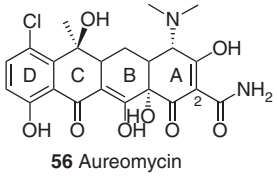




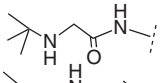
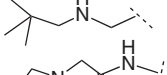
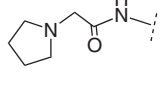
53 Rifaximin

54 Rifalazil R_1 = Ac R_2 = *i*-Bu

55 ABI-0043 R_1 = H R_2 = Me



56 Aureomycin

	R_1	R_2
57 Minocycline	NMe_2	H
58 Tigecicline	NMe_2	
59 Omadacycline	NMe_2	
60 TP-434	F	

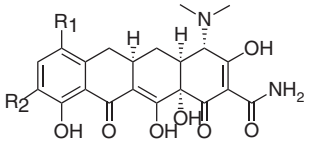


Figure 1.7 Macrociclic antibiotics, rifamycins, and tetracyclines.

first ketolide approved for clinical use in the 2000s. Further improvement led to cethromycin **46** and solithromycin **47** as lead compounds, currently under clinical development [3]. Both are characterized by the presence of a cyclic carbamate group at the 11, 12-position that enhances the activity against susceptible and resistant strains by stabilizing the ketolide conformation.

1.7.2 Difimicin

Difimicin **48** (Figure 1.7) belongs to an 18-member family of actinomycete-produced macrocycles, independently discovered under the names of lipiarmycin, clostomycin, and tiacumicin [13]. Compounds in the family differ for variations in the macrolide ring and for the nature and position of the acyl residue esterified on the sugars, with the major component carrying an isopropyl ester at position 4″. Among the natural congeners, lipiarmycin B **49** is less active than lipiarmycin A against all bacterial species, thus indicating that the position of the isobutyl residue on methyl rhamnose affects *in vitro* activity. Difimicin is a potent inhibitor of bacterial RNA polymerase (RNAP) and is currently under registration for the treatment of *C. difficile* infections.

1.8 Ansamycins–Rifamycins

The class of ansamycins is characterized by a cyclic structure in which an aliphatic chain forms a bridge between two nonadjacent positions of a cyclic π-system, similar to the handle of a basket or ansa (in Latin, hence the name). They are produced by strains of several genera of the order *Actinomycetales*. The most important ansamycins are rifamycins (Figure 1.7). They have an aliphatic ansa chain constituted of 17 atoms, are antibacterial, and selectively inhibit RNA polymerase. Following the first rifamycin isolation in 1957 (rifamycin SV **50**), extensive programs of semisynthesis led to the preparation and evaluation of a large number of rifamycin analogs with the aim of obtaining a compound with better oral absorption, more prolonged antibacterial levels in blood, and greater antimicrobial activity [30]. These studies gave important information on the SAR in rifamycins. The minimal requirements for antibiotic activity appeared to be the presence of the two hydroxyls at C_{21} and C_{23} positions of the ansa chain and the two polar groups at C_1 and C_8 positions of the naphtoquinonic nucleus, together with a conformation of the ansa chain that resulted in certain specific geometric relations among these four functional groups. Position 3 of the aromatic nucleus has been extensively derivatized, mainly starting from the readily available intermediate 3-formyl rifamycin **51** resulting in the synthesis of interesting compounds, among them rifapentin **52**, currently used for the treatment of tuberculosis in the United States, rifaximin **53**, and rifalazil **54**. Novel benzoxazinorifamycins have been recently synthesized and screened. Among them, novel derivatives (ABI-0043 **55**

is the main example) that possess both the ability to suppress the emergence of rifamycin-resistant mutants and show increased activity against mutants resistant to other rifamycins have been identified [30].

1.9 Tetracyclines

Tetracyclines are characterized by a polycyclic structure consisting of a highly functionalized and partially reduced naphthacene (Figure 1.7). They are usually produced by strains of *Streptomyces aureofaciens* and *Streptomyces rimosus* and, more recently, by *Micromonospora* and *Actinomadura brunea*. These molecules bind to the ribosome-inhibiting protein synthesis and are classified as broad-spectrum antibiotics. The first member of the group chlorotetracycline (aureomycin **56**) was discovered in the late 1940s. The first structural variations of the basic skeleton, obtained by semisynthesis from the natural precursor, were generally related to the C-5, C-6, C-7, and C-8 carbons and the carbamoyl group at position C-2 [31]. Since the 1970s, when minocycline **57** was approved, only tigecycline **58** has been introduced, in 2005, to treat infections resistant to other antimicrobials [32]. Nevertheless, the medicinal chemistry and semisynthesis of newer analogs have recently undergone a renaissance; moreover, total synthesis has become available, giving access to a broad range of tetracyclines that would be inaccessible by semisynthesis and provides a powerful engine for the discovery of new tetracyclines [33]. Among the new derivatives, omadacycline **59** (PTK-0796) is in phase III while TP-434 **60** is currently in phase II trials [3].

1.10 Oxazolidinones

Oxazolidinones are a new class of synthetic antibiotics, discovered in the 1980s (Figure 1.8) [18]. These compounds originated from an iterative medicinal chemistry effort starting with a series of racemic 5-halomethyl-3-phenyl-2-oxazolidinones with reported utility for treating a variety of plant diseases [34]. In 2000, the FDA approved linezolid **61**, which also showed a unique mechanism of protein synthesis inhibition [35]. Detailed SARs were obtained, leading to the identification of the molecular feature critical for the antibiotic activity. Linezolid is composed of an oxazolidin-2-one ring containing a critical (S) stereocenter in position 5. Aryl substitution of the nitrogen is also necessary for activity. Generally, the B-ring of oxazolidinone antibacterials contains a phenyl ring or fluorosubstituted phenyl rings. In addition to these, heterocyclic B-rings such as pyridine and pyrrole ring systems were also reported in the literature, showing limited improvements. In the most recent derivatives, additional rings C and D were introduced and/or modified following extensive chemical investigation. There are several oxazolidinone derivatives that are being clinically developed and several others that are in

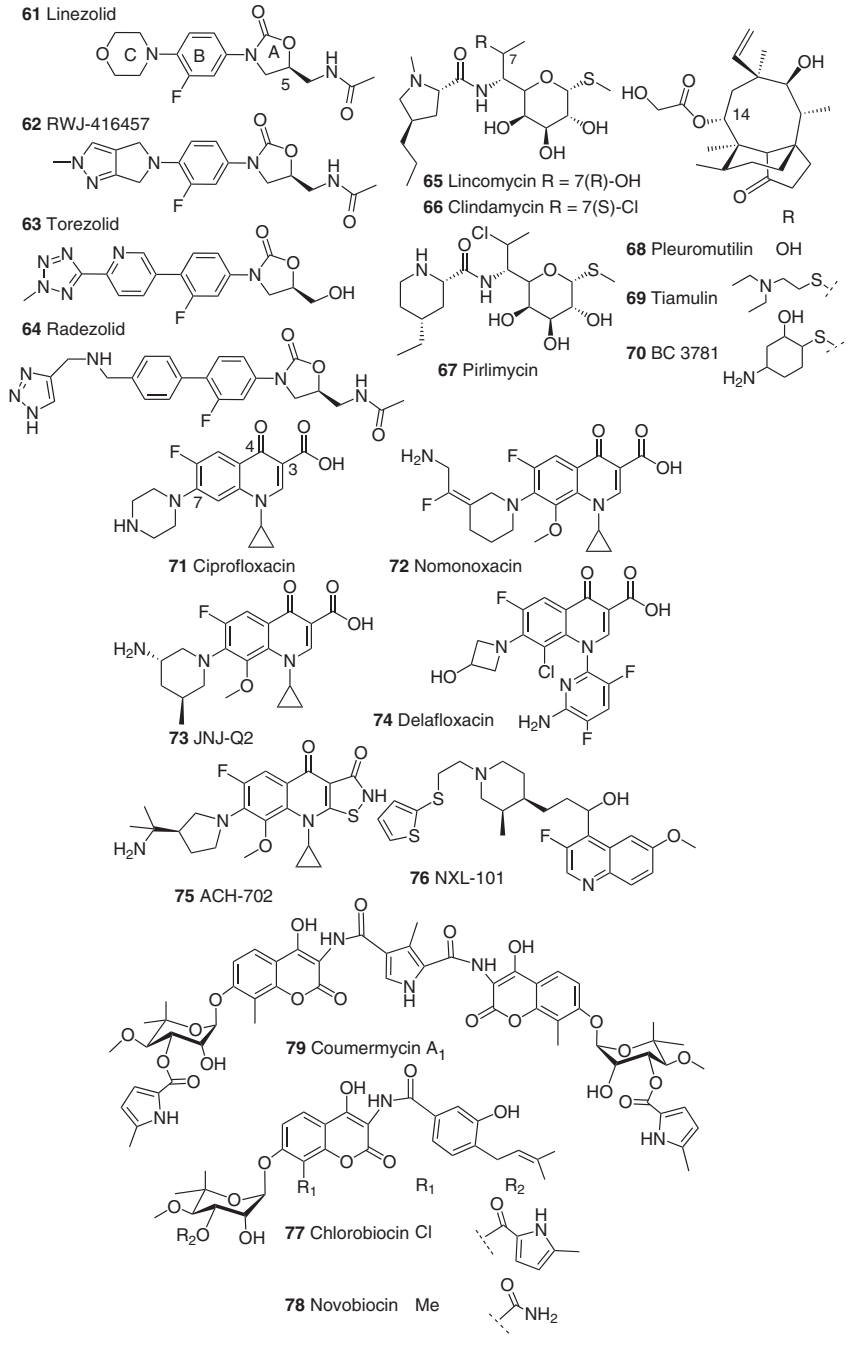


Figure 1.8 Oxazolidinones, lincosamides, pleuromutilins, and aminocoumarines.

preclinical development. Among the most interesting, RWJ-416457 **62** is modified on the C and D rings [36], torezolid **63** in addition to ring modification lacks the acetyl group [37], while radezolid **64** contains a new modification on the C-5 substituent [3].

1.11 Lincosamides

Lincomycin **65** (Figure 1.8) was isolated by fermentation of *Streptomyces lincolnensis* and was introduced in clinical medicine in 1960. Its semisynthetic analog clindamycin **66**, obtained by selective halogenation of the secondary alcohol in position 7, was also approved a few years later, in 1969 [4]. Lincosamides inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit and show activity against most gram-positive and anaerobic bacteria but not against gram-negative and enterococci. Their use was limited by development of resistance due to methylation of the ribosomal target causing reduced binding affinity. Recently, new medicinal chemistry research programs were started to achieve second-generation lincosamide derivatives. Among the new products, pirlimycin **67** possesses an improved pharmacokinetic profile even if no improvement was observed in its spectrum of action. The molecule has been marketed for veterinary use.

1.12 Pleuromutilins

The diterpene antibiotic pleuromutilin **68** (Figure 1.8) from the fungus *Clitopilus* sp. was first discovered in 1951 and inhibits protein synthesis [38]. A series of derivatives was synthesized between 1963 and 1966, with a strong focus on variations in the C(14) side chain. Already at that time, it was recognized that the number of functional groups was small, which led to the consideration that an ''activation'' of the molecule via sulfonic acid esters at the C(14) atom would give the best opportunities for numerous exchange reactions. Of the derivatives generated, the mutilin esters of substituted thioglycolic acids demonstrated superior minimum inhibitory concentration (MIC) values. Further alterations within this group led to the development of the first veterinary pleuromutilin, tiamulin **69**, which was approved in 1979. Despite successful use in veterinary medicine, no derivative for systemic use in humans has been made. More recently, with the dramatic emergence of resistance to established antibacterial classes in the 1980s, significantly more attention has been given to the class of pleuromutilins to try to explore their potential for human use owing to its unique interaction with the bacterial 50S ribosomal subunit. Side-chain chemistry, in combination with extensive use of SARs derived from more than 1000 pleuromutilin derivatives, has led to the discovery of BC-3781 **70**, the very first systemic pleuromutilin recently tested successfully in patients in a phase II trial [39].

1.13 Quinolones

The quinolones (Figure 1.8) are a family of synthetic broad-spectrum antibiotics. Nalidixic acid, introduced into clinic practice in 1962, is considered to be the predecessor of all members of the quinolone family. The basic structure consists of an N-1 alkylated 3-carboxypyrid-4-one ring fused to another aromatic ring, which may contain various substituents. Quinolones inhibit two essential bacterial enzymes: DNA gyrase and topoisomerase IV to varying extents depending on the pathogen. Substitutions vary by drug and may influence activity. The 1-position can include small alkyl groups or an aryl group. The 2-position will maintain activity when it is either a carbon or nitrogen atom. Positions 3 and 4 are critical and must not be altered from the unsubstituted carboxylic acid and ketone, respectively. Substitutions are usually made at positions 5, 6, 7, and 8 to improve activity. Most newly reported quinolones have modifications of the crucial 7-position (piperazine group for ciprofloxacin **71**). The majority of quinolones in clinical use belong to the subset fluoroquinolones, which have a fluorine atom attached to the central ring system, typically at the 6-position or C-7 position. In fact, fluorine, chlorine, and methyl all appear to show improvement, with 6-fluoro giving the most significant improvement [40]. Despite the fact that the use of quinolones has been associated with increased incidence of MRSA, several new members of this class are under development: nemonoxacin **72**, JNJ-Q2 **73**, delafloxacin **74** [6]. Related compounds isothiazoloquinolones (ITQs) were first described by Abbott 20 years ago. They are one of the few quinolone analogs in which the carboxylic acid has been successfully replaced. ACH-702 **75** belongs to this group [41]. Strictly related to quinolone are quinolines, in which the aromatic nucleus does not contain the oxidation in position 4. NXL101 **76**, currently in phase I clinical trials, is representative of a new class of quinoline DNA gyrase and topoisomerase IV inhibitors with a gram-positive spectrum of antibacterial activity including MRSA- and fluoroquinolone-resistant isolates [42].

1.14 Aminocoumarins

Aminocoumarins such as clorobiocin **77**, novobiocin **78**, and coumeramycins produced by *Streptomyces* sp. are known inhibitors of GyrB ATPase (Figure 1.8) [43]. Their common characteristic structural moiety is a 3-amino-4,7-dihydroxycoumarin ring, substituted at position C-8 either with a methyl group or with a chlorine atom. In all three compounds, the 7-hydroxy group of the aminocoumarin moiety is glycosidically linked to an unusual deoxy sugar, 4-O-methyl-5-C-methyl-l-rhamnose, which is acylated at its 3-hydroxy group with a 5-methylpyrrole-2-carboxyl group or a carbamyl group. Coumermycin A_1 **79** is unique among the aminocoumarins in incorporating two 3-amino-4,7-dihydroxycoumarin moieties, which are connected through amide bonds to a central pyrrole unit-3-methylpyrrole-2,4-dicarboxylic

acid, resulting in a nearly but not completely symmetric molecule. Novobiocin was once marketed as an antibacterial but has since been withdrawn owing to toxicity. Several papers from Heide and colleagues over the past few years report new novobiocin analogs that have been isolated by selective manipulation of the biosynthetic gene clusters of *Streptomyces* [44].

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2
Antibacterial Discovery: Problems and Possibilities

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2.1
Introduction

The approach of selecting single enzymes as candidates for inhibition and development into antibacterial agents has not proved as successful as hoped, and actually has been rather a failure [1–3]. Discovery of novel antibacterials is not a simple stepwise linear process. It cannot simply start with a novel unexploited target *in silico* or in reality and proceed with design of or screening for inhibitors without taking into account the ability of any such inhibitor to enter (and avoid efflux from) the appropriate bacterial cells and the high probability that a selective inhibitor of any single-bacterial enzyme will select rapidly for resistance (in a sufficiently large population of bacteria). The physicochemical parameters correlating with cell entry and efflux avoidance should be kept in mind during any optimization process. In that regard, the spectrum of an antibacterial agent is based both on the presence of homologous targets across a useful set of bacterial species and on the the permeability barriers present in these species.

It is clear that the problem of rising antibacterial resistance leading to reduction of the efficacy of most of the standardly used antibacterial agents has led to anxiety that we may soon enter a new post-antibiotic era. In attempting to address this, many interest groups and agencies have responded by conjuring push/pull incentives for Big Pharma to get back into the antibacterial drug discovery or development business. This seems to be based on the idea that Big Pharma deemphasized antibacterials largely based on financial concerns and the regulatory difficulties of developing new agents. And undoubtedly this is in part true. But it is also true that Big Pharma had worked on antibacterial discovery until the mid-2000s, using all the tools of genomics, high-throughput screening (HTS), bioinformatics, combinatorial chemistry (when that was in vogue) – and yet, no novel class that has been registered was discovered after 1987. Antibacterial drug discovery is difficult. It has not been conquered, as many in the early 1990s predicted, by identifying new targets, screening for, and finding novel inhibitors.

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2.2 Why Is Antibacterial Discovery Difficult? The Problems

Review of many programs based on *in vitro* inhibition targets shows that although inhibitors may be discovered through screening or design, most programs stop prematurely – long before identification of a clinical candidate. It is never quite clear why such programs do not progress but major bottlenecks are the lack of antibacterial activity, even against highly permeable bacteria, despite extensive medicinal chemistry efforts; the mistaken (and eventually misguiding) attribution of any antibacterial activity to the inhibition of the initial enzyme of interest; and a relatively high frequency of high-level resistance. And these are independent of the need for spectrum based on the presence of homologs in desired bacterial species, potency, useful pharmacokinetics (PK), low protein binding, and other absorption, distribution, metabolism, and excretion (ADME) properties. Oral bioavailability – which is a major requirement in much of human health drug discovery – is not so critical for development of drugs for therapy of highly resistant bacterial infections, as they are generally treated in a hospital. With the possibility of IV dosage (or its frank benefit), antibacterial drugs may be allowed to have and would – it turns out – benefit from physicochemical parameters that differ from standard drugs. Thus, the particular difficulties of antibacterial discovery lie in target choice and cell entry – as discussed here.

2.3 Target Choice: Essentiality

Much of antibacterial discovery since the advent of the genomic era has consisted of cataloging and prioritizing targets on the basis of several criteria. Important to ensuring coverage of pathogens necessary for a specific indication is the spectrum of organisms in which the target is present, and this can be assessed by sequence and bioinformatics analysis. Similarly, selectivity for pathogen over host is important, so bioinformatics can be used to set requirements for differences between bacterial and mammalian homologous proteins. Often, criteria for target choice include the existence of X-ray crystallographic data so that inhibitor chemistry and enzyme structure can be correlated or even predicted. Information on structural variation in putative target enzymes across bacterial genera is also important for target prioritization. Whether targets are ''druggable,'' able to be inhibited by small drug-like molecules, is a question discussed more recently – especially in light of the dearth of leads found for inhibitors of antibacterial targets (see subsequent text).

The crucial requirement for an antibacterial target is its essentiality for growth and, preferably, viability of the pathogen. While essentiality of bacterial proteins was standardly demonstrated in the early days of microbial genetics by studying conditional mutants where the effects of inactivating a function could be measured in real time, the determination of essentiality nowadays is generally deductive, in that it is based on large-scale efforts to delete or inactivate genes or gene products

under a given set of growth conditions. Usually, those growth conditions are relatively nutrient rich to prevent identifying auxotrophs. But are those conditions, at least in terms of nutrients, similar to what will be seen in the host? Will the host provide for all auxotrophs? A study on the growth requirements of auxotrophic *Escherichia coli* that are not met in human blood shows that intact bacterial pathways of nucleotide precursor synthesis are the most critically required for growth in blood [4]. That is, purines and pyrimidines are limiting for the growth of auxotrophs in their synthetic pathways. Thus, certain genes identified as inessential *in vitro* (in rich media) may be essential *in vivo*.

On the other hand, will certain proteins be incorrectly deemed essential because the host *can* provide the needed supplement? This was proposed for enzymes of fatty acid synthesis (which are normally thought to be essential and good antibacterial targets) in gram-positives, based on experiments showing that fatty acids present in blood could overcome inhibition of the pathway, at least in *Streptococcus agalactiae* [5]. Later work argued against this finding and showed that inhibitors of FabI in *Staphylococcus aureus* were not reversed by fatty acid addition [6]. As discussed by Parsons and Rock [7], the particulars of uptake and incorporation of exogenous fatty acids may be different for different organisms, these differences are not sufficiently well studied, and the success of experiments showing efficacy of fatty acid synthesis inhibitors in animal models should heavily counter a blanket caveat against such targets.

The study of *in vivo* essential genes, those required only for growth in the host, pioneered by Mekalanos *et al.* (reviewed in [8]) can detect genes involved in colonization and growth and will pick up the above-mentioned essential auxotrophs as well as functions involved in adherence, survival in the presence of the immune system, and many other functions. Such *in vivo* essential genes have long been proposed as targets. As these functions are, by definition, required for growth in the host, inhibitors of such functions would then be expected to select for resistant mutants whose growth is not impaired. Thus, as will be discussed later, these targets would be subject to the same resistance caveats as the standard *in vitro* essential single-gene targets. But they should probably be viewed separately from the so-called antivirulence or antipathogenesis targets. A recent review by Hill [9], who writes from the point of view of one interested in the microbiome and probiotics, argues that there are what he calls ''niche factors ... that ... are often shared by harmless commensals sharing the same body site'' that ''promote colonization and survival,'' which should be distinguished from virulence factors ''that cause damage to the host.'' And, it does seem likely that inhibitors of functions solely involved in host injury would be less apt to select for resistance than inhibitors of niche factors. On the other hand, as antiniche therapeutics would most likely be used in combination with a standard antibacterial, resistance-selection potential might be minimized.

While there have been many academic programs investigating antivirulence targets (of both types), as well as some small companies (Athelas, Mutabilis), Big Pharma has not been seriously involved in this pursuit. This is most likely because of the likelihood of narrow spectrum and the perceived difficulty of

developing such agents, even as adjuncts to standard antibacterial therapy. For one thing, development could be hampered by the difficulty of assaying for susceptibility to such agents in a population of pathogens as there would likely be no simple MIC-like measurement that could be used. However, as the field of rapid diagnostics is growing, it is likely that such susceptibility tests for antivirulence/niche factor therapy could be designed. Thus, if there were a clear regulatory path for development these targets should be considered.

2.4 Target Choice: Resistance

Many reviews and discussions of antibacterial resistance are based on resistance to the standardly used monotherapeutic agents (see Table 2.1 for a list of classes of clinically used classes of antibacterial agents), on the epidemiology of resistance mechanisms, their spread, both by horizontal genetic transfer (HGT) and by

Table 2.1 Targets of clinically used classes of antibacterial drugs.

Drug class	Pathway	Target	Use
A. Multitarget			
β-lactams	Cell-wall synthesis	PBPs	Systemic monotherapy
Glycopeptides	Cell-wall synthesis	Lipid II	Systemic monotherapy
Fluoroquinolones	DNA replication	DNA Gyr/Topo IV	Systemic monotherapy
Aminoglycosides	Protein synthesis	16S rRNA	Systemic monotherapy
Tetracyclines	Protein synthesis	16S rRNA	Systemic monotherapy
Oxazolidinones	Protein synthesis	23S rRNA	Systemic monotherapy
Macrolides	Protein synthesis	23S rRNA	Systemic monotherapy
Lincosamides	Protein synthesis	23S rRNA	Systemic monotherapy
Streptogramins	Protein synthesis	23S rRNA	Systemic monotherapy
Chloramphenicol	Protein synthesis	23S rRNA	Systemic monotherapy
Metronidazole		DNA	Systemic monotherapy
Polymyxin		Cell membrane	Systemic monotherapy
Daptomycin		Cell membrane	Systemic monotherapy
Pleuromutilin	Protein synthesis	23S rRNA	Topical therapy
B. Single target			
Trimethoprim	Folate synthesis	DHFR (FolA)	Systemic in combination
Sulfamethoxazole	Folate synthesis	FolP	Systemic in combination
Rifampicin	RNA synthesis	RNA polymerase	Systemic in combination
Mupirocin	Protein synthesis	Ile tRNA synthetase	Topical therapy
Fosfomycin	Cell-wall synthesis	MurA	Systemic UTI
Fusidic acid	Protein synthesis	Ef-G	Systemic UTI
Fidaxamicin	RNA synthesis	RNA polymerase	Oral (nonabsorbed) for *C. difficile*

rRNA, ribosomal RNA; DHFR, dihydrofolate reductase; and Ef-G, elongation factor G.

resistant clones, and the overall nature of the resistome – the totality of genes that contribute to resistance to antibiotics [10]. How does an understanding of the resistome affect drug discovery and target choice? The intimation that resistance will inevitably arise to whatever drug we fashion is daunting. So, let us step back, to widen our focus on the problem of drug resistance to other therapeutic areas. In viruses such as HIV and hepatitis C virus (HCV), it is now recognized that mutations conferring resistance to small molecule drugs targeting viral gene products are preexisting (before drug challenge) in a population of viruses [11–15] and will lead eventually to therapeutic failure. And this may be true of cancer as well, at least in some cases, such as chronic myeloid leukemia (CML) and epidermal growth factor receptor (EGFR)-mediated colorectal tumors [16–18]. This recognition of the probability of rapid, often preexisting, resistance to single-target inhibitors has given rise to the practice of using combinations of drugs, which are not cross-resistant, to combat HIV, HCV, and cancer, as well as *Mycobacterium tuberculosis* (MTB).

But resistance to standard antibacterials is thought of differently. It is thought to arise relatively slowly over time, by stepwise changes due to adaptive epigenetic mechanisms, gene amplification, mutations in the pathogen leading to incremental increase in MIC, and by HGT [19]. Standard antibacterials have, indeed, been subject to increased resistance due to these factors – but that increase has generally taken some time and does not usually rise rapidly to clinical significance. This is true because the monotherapeutic systemic drugs that are the backbone of clinical therapy are not prone to preexisting, high-level, single-step resistance. They are *not* targeted at single-gene products – as are the antivirals. Almost all of them are ''multitargeted'' (Table 2.1A). Multitargeted, in this sense, means that multiple genes encode the target or targets or encode the proteins that produce the target. This sense of targeting is at the genetic level. It is genes that are subject to mutation – and if multiple genes are involved in the process, single changes cannot change all targets. The concept of the benefits of multitargeting of antibacterials has been discussed in several of my papers and those of others [20–25].

If we choose as targets the products of single genes, the output of genomic and bioinformatic searches, then it seems highly likely that the same sort of preexisting resistance mutations (as seen in viruses) will be selected for drug treatment and could, under the right circumstances, lead *overnight* to high-level resistance and even failure of therapy. In my previous discussions on the subject of the likelihood of single targets being subject to rapid resistance [2, 21], the idea that these rapidly arising resistance mutations were most likely preexisting was not made explicit, but it was my expectation as a microbial geneticist – and is reflected in the recommendations of myself and others, that the propensity of antibacterials to select for resistance should be tested in a fluctuation test, à la Luria and Delbruck [26]. The fluctuation test was proposed specifically as a way to prove that mutations can be preexisting and do not require the presence of a selective agent – and also can give a measure of the rate of resistance mutations per generation (before being challenged by drugs). Over the years, variations in the methodology have been

proposed, but the goals of showing that mutations are preexisting and determining rate have remained.

The benefits of and methodology for doing a fluctuation test have been recently reviewed [27–29]. In simple terms, the fluctuation test involves growth of a series of tubes (e.g., 21) of bacteria in a small volume of medium, starting from a small inoculum (e.g., 10^3 bacteria/tube) – such that there is no likelihood that the inoculum contains a resistant mutant. The tubes are incubated until they reach a concentration around that of the inverse of the resistance frequency (estimated on the basis of preliminary experiments in which a large known inoculum ($\geq 10^{10}$) on selective drug). Thus, for the fluctuation test, if the frequency is 1×10^{-8}, then the contents of 20 of the tubes are plated when they reach somewhat greater than 10^8 total bacteria to be plated on the selective drug. The twenty-first tube is plated on a nondrug plate to determine the titer. If mutations to resistance occur only when the bacteria are in the presence of the drug, then all tubes would produce a similar number of resistant colonies on each of the drug plates. However, when mutations occur before drug exposure, there will be a great variation in the number of colonies on the plates. Many plates will have no colonies, while others will have a range of colony numbers. This variation, or fluctuation, is due to the occurrence of mutations during the generations of growth before drug challenge. Mutation occurring earlier will give rise to more progeny (through geometric growth of each mutant), and those occurring later have fewer progeny; in a proportion of tubes, there will be no colonies. In fact, the average rate of mutation to resistance can be determined from the number of tubes containing zero colonies.

During therapy, then, preexisting resistant mutants will be present if there is a sufficiently large bacterial burden. If the magnitude of the resistance, the increase in MIC, is higher than the C_{max} *in vivo*, then these resistant mutants are likely to survive. Although they may have reduced fitness relative to sensitive bacteria, they will be more fit than their dead brethren. Success or failure of therapy is hard to predict a priori, as not only fitness but virulence of the resistant mutants will also play a role. This can be modeled *in vitro*, for example, in a hollow-fiber model [30] or *in vivo* using sufficiently high infectious burden. There has been little published on the use of such models for novel single-targeted agents, yet, standardized testing of a wide variety of antibacterials, especially single-targeted ones, should start to give a pattern relating *in vitro* resistance rate and fitness to *in vivo* survival of bacteria and success or failure of therapy. It should be noted that most tests of efficacy in animal models of bacterial infection are done at quite low inocula – very different from the case in a fulminant infection. While little has been published on *in vitro* and *in vivo* modeling of resistance development of single-targeted agents in standard pathogens, quite a bit of work has been done with such agents used against *M. tuberculosis* – and lessons learned from this work could be profitably applied to more standard pathogens. All anti-MTB drugs so far are single targeted and resistance is known to arise to each of them via single-step endogenous mutations. This is a major reason why MTB is treated with combinations of these agents (although there are additional reasons involving the need to kill the pathogen living in multiple states). A number of studies modeling resistance development and the

dosing regimens and/or combinations that could be used to minimize resistance selection have been published by Gumbo and Drusano [31–35].

If antibacterial discovery is turned solely to finding inhibitors of single-enzyme targets, then it seems likely that the same pattern of resistance seen in viruses and cancer would be expected – and there would be a necessity to treat with combinations of these single-targeted agents in order to prevent therapeutic failure due to preexisting mutations. Or, initial dosing at very high levels to reach a mutant prevention concentration (MPC) could serve to kill off the initial resistant mutants.

A very real approach to the problem of single-targeted agents is the use of iterative design and testing to produce inhibitors which, by dint of having additional enzyme binding sites, can overcome resistance mutations. The DHFR (dihydrofolate reductase) inhibitor trimethoprim (Figure 2.1a) selects for mutations in DHFR and analogs of trimethoprim that act against the resistant enzyme have been made, the prime example being iclaprim (Figure 2.1b) [36]. Iclaprim binds with approximately equal affinity to trimethoprim-sensitive and trimethoprim-resistant enzymes and has very low resistance selection potential itself. This illustrates how an established drug can be redesigned to overcome specific preexisting resistance – but novel discovery of single-target inhibitors could take into account resistance potential in initial design. In that regard, Anderson and coworkers [37] have recently approached design and discovery of DHFR inhibitors with prospective screening of resistance potential. They investigated the resistance potential of two of their leads (Figure 2.1c,d), the fitness of the mutants, the kinetic properties of the mutant enzymes, and the MPC – finding that, while compound 1 (Figure 2.1c), with an MIC of 0.076 $\mu g\,ml^{-1}$, selected for resistance at low frequency (1.2×10^{-9} to 6.9×10^{-10}, depending on the mutation selected), the MPC concentration was relatively low as well ($\sim$1 $\mu g\,ml^{-1}$). Conceivably, these compounds could be iteratively optimized further for lowered resistance frequency and MPC.

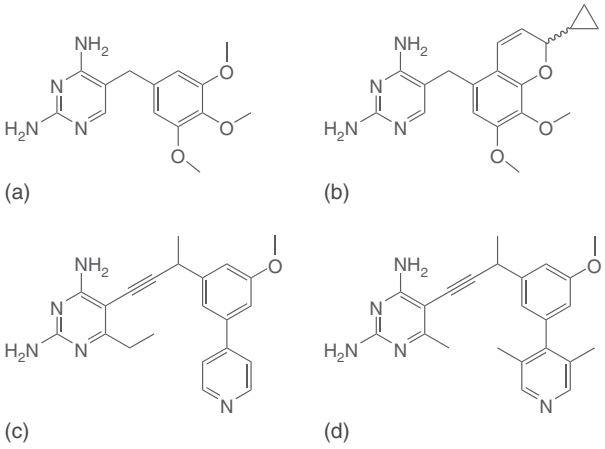

Figure 2.1 Dihydrofolate inhibitors designed to overcome resistance. (a) Trimethoprim, (b) iclaprim, and (c,d) compounds 1 and 2, respectively, of [37].

With bacteria, single-enzyme targets are, in actuality, a set of enzyme orthologs (and paralogs), the enzymes of all the bacterial species in the proposed spectrum; thus, an inhibitor would have to hit all of the orthologs. For inhibitors designed to have high affinity due to multiple interactions in order to avoid resistance, it seems likely that the spectrum could be reduced. For cancer or for a virus, on the other hand, where the enzymes are species specific and only one species causes the disease (in cancer, this is homo sapiens), such engineering of inhibitors to have strong and multiple sites of interaction with the target enzyme should be more successful. This was done in the design and discovery of ponatinib by Ariad Pharmaceuticals [38], a second-generation inhibitor of the BCR-ABL kinase, which is responsible for CML (and a smaller percentage of acute lymphocytic leukemia). The first such inhibitor, Gleevec (imatinib), has been a highly successful drug and revolutionized CML therapy and prognosis, but Gleevec-resistant mutations in the kinase do arise. Ponatinib was specifically designed to be active against the major and minor mutant BCR-ABL kinases and looks promising in the clinic.

Of course, the rates of mutation and the infectious burden vary among viruses, bacteria, and fungi. For the RNA viruses HIV and HCV, the mutation frequency is very high. In an experiment with simian-HIV-infected macaques, certain efavirenz-resistant mutations were detected at a frequency of 1.4×10^{-4} in the viral population after 1 week of infection (before treatment) [14]. The infectious burden for HIV is a subject of contention and it is likely that the number of cells in which the viruses replicate should be considered, which may be as high as 10^6 [14] with the actual number of HIV virions being produced at $\sim 1 \times 10^{10}$ per day [39]. For fungal and bacterial infections, organismal load is generally calculated (often from polymerase chain reaction, PCR, techniques) on the basis of colony-forming units per milliliter (cfu ml^{-1}) of homogenized sample or per gram of material (such as an endocarditis vegetation), with total body burden not usually indicated. For Candida and Aspergillus infections, a range of concentrations has been seen, most in the (remarkably low) range of ~ 10 cfu ml^{-1}, with some as high as 100 cfu ml^{-1} [40]. A fluctuation test to determine the rate of resistance of *Candida albicans* to an echinocandin (resistance due to mutations in glucan synthase) yielded a rate of 2×10^{-8} [41], on the order of resistance frequencies seen for single-gene targets in bacteria. Bacterial burdens in various infections have been measured: in soft tissue and intra-abdominal infections of humans, the burden was, on average, 10^8 cfu ml^{-1} [42]; in animal models of gram-positive endocarditis, vegetations contained from 10^8 to 10^{11} cfu g^{-1} (quoted in [42]). As the appearance of mutations in an infected individual (before treatment) would be related to the burden times the inverse of the frequency of resistance selection, it can be seen that for the RNA viruses, with a high burden and extremely high resistance frequency, such resistance mutations are prevalent. For bacteria, the burdens may be high, but frequencies are lower than for viruses; therefore, the prevalence of pre-existing resistant mutants should be lower. Even though the frequency of mutations in the above-mentioned fungal experiment is in the same order as that seen for bacteria, the organismal load of fungi is drastically lower than for bacteria – and, indeed, it

is clear that single-step mutations to antifungal resistance are rarely seen during therapy.

It has been my contention that the high probability that inhibitors of single-enzyme targets will select rapidly for resistance, generally target based, and thus any selected single enzyme as target will have a strike against it from the outset will only be removed after a selective inhibitor is discovered, with which to prove that spontaneous, single-step resistance does not arise [21]. Unfortunately, the most convincing argument for an inhibitor being selective for a single target is its ability to show that resistance to that inhibitor maps to the target gene. Novel antibacterial discovery is driven by the slow but inexorable rise in resistance to the standard monotherapeutic antibacterials on which therapy has depended, which is generally due to HGT. However, the probability that novel discoveries might be subject to more rapid selection of resistance is not sufficiently taken into account. Recently, a phase II clinical trial of a novel inhibitor of gram-negative leucyl-tRNA synthetase, GSK2251052, was suspended and development later halted because of resistance occurring during therapy. Publication of this study will be very helpful for thinking about the resistance potential of single-targeted drugs.

2.5
Cell Entry

A critical factor in the discovery and development of antibacterial agents is the need to get the inhibitor to its site of action, its target. This is most problematic for gram-negative bacteria, which are bounded by two membranes having orthogonal sieving properties: the inner, symmetric, bilayer cytoplasmic membrane that favors diffusion by neutral, relatively hydrophobic compounds and the outer, asymmetric, membrane that favors entry of hydrophilic, polar, and preferably charged molecules via water-filled channels called *porins*. In addition, efflux pumps with broad substrate specificity (that translocate small molecules from the periplasm or outer leaflet of the cytoplasmic membrane to the medium) are ubiquitous among gram-negatives and can act synergistically with the outer membrane permeability barrier to reduce cellular accumulation of drugs. The problem is exacerbated in the lactose nonfermenting gram-negatives, such as *Pseudomonas aeruginosa*, that have very low outer membrane permeability, coupled with the genetic capacity for expression of many efflux pumps of various substrate specificity [43]. The roles of membranes and efflux in antibacterial resistance have been explored in many reviews, among them [44–48]. While there are no fixed rules for the properties that endow molecules with the ability to enter gram-negatives, relatively recent analyses of the physicochemical characteristics of antibacterials grouped by their spectrum (gram-negative or gram-positive) and target location (cytoplasmic or extracellular/periplasmic) have appeared [47, 49, 50], and further exploration and experimentation in this area should lead to development of a better understanding of requirements for entry [51].

In the absence of rules for entry, are there other means that might be explored for overcoming permeability barriers and efflux? A recent paper outlines a number of approaches [52], among them adjunctive molecules that lead to cell permeabilization (such as polymyxin B nonapeptide, PMBN), efflux inhibitors, and the possibility of some sort of nanoparticle packaging that would allow or promote cell entry.

2.6 Screening Strategies

Some definitions first. Empirical screening here means screening for chemicals that kill or inhibit growth of bacteria, without taking into account mechanism of action. Phenotypic screens, more specifically, are whole-cell screens that detect the inhibition of a desired target or function, in a way analogous to selecting genetic mutants by their demonstration of an expected phenotype. *In vitro* assays include, obviously, inhibition of enzymes, binding of compounds to enzymes, inhibition of critical interactions, and can have a variety of readouts and platforms. Most of present day screening seems to be via HTS mechanisms which favor *in vitro* screens – but can be and have been accommodated to whole-cell empirical and phenotypic assays.

It is difficult to divorce the nature of the chemicals screened from the optimum strategy for screening them. The screening scenario must match the screen to the source. Thus, the discussions in subsequent text, while separated into ''screening modes'' and ''chemicals to screen'' will involve considerable mixing.

2.6.1 Empirical Screens

Table 2.2 shows the strategies and sources of classes of antibacterials in clinical use. It is obvious that the rational discovery strategies employed since the 1960s have not been very productive in bringing drugs to market. The successful era of empiric screening – that is, screens for inhibition of cell growth without regard to mechanism of action – was coincident with the Golden Age of natural product (NP) discovery (from the 1940s to the late 1960s). And most classes of antibacterial drugs for human and animal health were indeed discovered by empirical screening of NPs, very often freshly fermented broths or extracts (as opposed to prepared libraries).

While empirical screening among NPs was productive for many years, the percentage of novel compounds found eventually decreased and the great majority of hits were identified as previously seen ''knowns.'' Powerful mechanisms of dereplication are needed to detect novelty among the prevalent previously seen activities. In the early days of empirical NP screening, this was often done by biological tests (comparison of the bacterial spectrum and potency of fermentation broths and extracts as well as purified compounds to a database of knowns) [53]. Interestingly, a recent publication presents a modernized version

Table 2.2 Sources of clinically used classes of antibacterial drugs.

Drug class		Source	Discovery strategy
β-lactams	NP	Fungi, actinomycetes	Empirical; also by spheroplasting (cell wall)
Glycopeptides	NP	Actinomycetes	Empirical
Fluoroquinolones	Synthetic	Quinine analogs	Empirical
Aminoglycosides	NP	Actinomycetes	Empirical
Tetracyclines	NP	Actinomycetes	Empirical
Oxazolidinones	Synthetic	Industrial library	Empirical
Macrolides	NP	Actinomycetes	Empirical
Lincosamides	NP	Actinomycetes	Empirical
Streptogramins	NP	Actinomycetes	Empirical
Chloramphenicol	NP	Actinomycetes	Empirical
Metronidazole	Synthetic	Azomycin analogs	Empirical
Polymyxin	NP	Bacillus	Empirical
Daptomycin	NP	Actinomycetes	Empirical
Pleuromutilin	NP	Fungi	Empirical
Trimethoprim	Synthetic	Pyrimidines	Target-based for DHFR inhibition
Sulfamethoxazole	Synthetic	Azo-dyes	Empirical in limited library
Rifampicin	NP	Actinomycetes	Empirical
Mupirocin	NP	Pseudomonas	Empirical
Fosfomycin	NP	Actinomycetes	Phenotypic by spheroplasting (cell wall)
Fusidic acid	NP	Fungi	Empirical
Fidaxamicin	NP	Actinomycetes	Empirical

NP, natural product.

of this approach [54]. The current prevalent methods involve rapid chemical fractionation and identification [55] and comparison to databases of known NPs.

In general, if empirical screening of NPs is done under conditions where strong growth inhibition is required, it is highly likely that novel compounds will be found very rarely – as the more common potent activities will already have been explored. Baltz estimates the chances of finding any individual new antibacterial antibiotic is 10^{-7} per Actinomycete screened [56]. There are an estimated 10^3–10^4 known antibacterial antibiotics known, so if there are a similar number yet to be found, the hit rate for finding any of these by random screening would be 10^{-3} to 10^{-4}, between 0.1 and 0.01%. As there is such a low percentage of novelty among NPs and dereplication can be time consuming and labor intensive, empirical screening of NPs has fallen out of favor. Historically, it was the decreased output of empirical screening that led to the advent of phenotypic screening (see subsequent text).

Empiric screening of chemical libraries has become more prevalent because target-specific *in vitro* screening has proved disappointing (see subsequent text)

and a few reports and reviews of the efficiency of this process have appeared. A study by workers at GSK [1] reported that an empirical screen of 500 000 synthetic compounds gave no exploitable hits against *E. coli* and, while there were thousands of anti-*S. aureus* activities, only 300 met the GSK requirements for hit progression: having activity against *S. aureus* plus one gram-negative or other gram-positive and being chemically tractable. The great majority of these 300 were ruled out as or being membrane active, alkylating agents, or to have other nonspecific activities. Another interesting study [57] gave the results of screening a diverse chemical collection of 150 000 small molecules for growth inhibition (60% inhibition at 12.5 μM) of *E. coli* or *P. aeruginosa*. The hit rates on *E. coli* and *P. aeruginosa* were 0.025% (i.e., 38) and 0.005% (or 8), respectively. The toxicity or lead potential of these hits is unknown.

In order to wade through false positives found in empirical screening of chemical libraries, a series of counterscreens are generally run to eliminate or deprioritize problematic compounds, including tests of cytotoxicity, red blood cell lysis, DNA binding, and serum protein binding. In this regard, serum protein binding is not itself exclusionary at this stage; but high serum protein binding can obscure cytotoxicity. Secondarily, a dose–response test for selective inhibition of macromolecular synthesis can both inform on possible mechanism of action and deprioritize those compounds that inhibit incorporation of all radioactive tracers within a narrow concentration range (which is often indicative of membrane depolarization or an energy poison). The few remaining hits can then be evaluated for mechanism of action by various mechanisms (transcriptomic, antisense, proteomic and other arrays, genetic selections for resistance, or protection by target overproduction [2, 58–60]) and characterized for spectrum and various pharmacological properties – in order to choose leads and goals for chemical optimization.

A home truth of empirical screening is that it is easy to kill gram-positive bacteria, even methicillin-resistant *Staphylococcus aureus* (MRSA) highly resistant to multiple other antibacterial classes. Empirical screening of any library, synthetic or NP, will find a great many initial hits – but most of them will be uninteresting, toxic, nonspecific or detergents – as the GSK group found. Among bacterial NPs, there will also be toxic compounds, but the large majority of hits will be selective antibacterials of known classes. So, empirical screening of chemical libraries will detect a very high percentage of false positives (uninteresting activities), while empirical screening of bacterial NPs will yield a very high percentage of knowns. Thus, simple kill-the-bug screens in both types of standard libraries will require powerful secondary and counterscreens.

2.6.2 Phenotypic Whole-Cell Screens

The bounty of empirically discovered antibiotics during the "Golden Age" of NP discovery was followed by study of their mechanisms of action, and empirical screening gave way to screening for new agents – still largely among NPs – by directed whole-cell screens designed to be selective for inhibitors of certain pathways, for new

members of old classes, and for new targets, as explained in subsequent text. But phenotypic screening was also an important tool for dereplication of NPs. While hits from empirical screening of NPs had to be differentiated at an early stage from the entire set of previously discovered antibiotics in order to avoid repetitive and unproductive chemical fractionation and purification, hits from phenotypic screening could be compared to other known inhibitors or other hits with the desired phenotype. Compounds chemically or biologically different from this shorter list of comparators could then be characterized. While some new hits could be previously known NPs, these might at least be reevaluated for their mechanism of action and could also be added to the list of comparators.

Phenotypic screens were designed to discover new classes of compounds with known phenotypes or phenotypes projected from observation of conditional mutants of essential genes. An example of the former is the spheroplasting screen for cell-wall-active agents, based on the behavior of penicillin-treated gram-negatives in hypertonic medium [53, 61]. The latter approach is exemplified by the search for inhibitors of DNA replication proteins, where temperature-sensitive mutations in those genes are known to lead to induction of the SOS pathway; hence, a screen for induction of the SOS response should find inhibitors of DNA replication – as well as DNA-damaging agents [62]. Several recent reviews discuss various types of phenotypic screens [53, 62, 63].

With the advent of tools for relatively easy genetic manipulation and engineering, bacterial strains could be manipulated to give enhanced readouts for interference with a specific intracellular function, gene product, or promoter. For example, reporter genes such as β-galactosidase (β-gal) or green fluorescent protein (GFP), or luciferase could be fused to any promoter and compounds causing induction of that promoter would be easily detected by measuring the amount of the reporter produced. Such promoters could regulate stress regulons – such as the SOS system responding to DNA damage noted earlier, or specific genes that can be correlated with inhibition of specific cellular pathways. Sets of such pathway reporters have been described [64–68]. These screens can be efficiently run in agar diffusion assays, where a test compound produces a gradient due to diffusion from a well or filter placed on agar inoculated with a test organism. After incubation, a zone of inhibition will surround the well or filter. This is useful because in assays such as the reporter-type screen noted earlier, high concentrations will kill or inhibit growth and thus reporter expression can be detected only at subinhibitory concentrations. In such agar diffusion screens, a zone of inhibition indicating frank antibacterial activity will be surrounded by a ring (at a lower concentration of compound) of reporter expression, be it blue (for X-gal hydrolysis by β-gal), fluorescent (for GFP), or light (luciferase). In a liquid assay, usually run at a single concentration, the critical subinhibitory concentration inducing the reporter, but not killing the cell, might be missed. In any screening format, these reporter assays have the benefit of demonstrating activity of the compound at much lower concentrations than are needed to inhibit growth – thus, they are hypersensitive screening methods.

In any of these reporter assays, it is useful to have a control strain or strains run in parallel to ensure that any hits are specific for the desired phenotype. With a bank of stress regulon screens, each strain will be a control for the others. With a reporter-based single target or pathway, a control strain should be constructed in which the chosen reporter is under the regulation of a different promoter, which should not be affected by the desired inhibitor. Also, a large test set of antibacterials with known modes of action should be run to confirm that the desired phenotype is not, in fact, producible by other means – such as general inhibition of transcription or translation. It is best to set up such phenotypic screens in a way as to give a positive readout. That is, looking for specific turn-on of a reporter is much better than looking for inhibition of its expression – as inhibition could be due to indirect inhibition via effects on general gene expression, other macromolecular synthesis, or even general membrane and lytic effects. In order to be robust, a screen must be well controlled and shown to have a very low rate of false positives. When HTS is used, there is a tendency to screen in duplicate with a single test and then follow up with a counterscreen ensuring specificity. I would favor primary screening with both tests. For specific novel antibacterial inhibitors of enzymes or pathways, the expected hit rate is very low. Were it not, empirical screening and follow up of most libraries would likely have found them already. Thus, if a screen has a 1% hit rate in a chemical library, it is highly likely that it is finding junk.

In fact, hypersensitive phenotypic screening methods, such as the reporter screens noted earlier, may be the key to a revival of NP screening – as their use has recently been shown to find interesting novel activities from standard Actinomycetes. Two methods should be highlighted: whole-cell antisense screens for specific targets and pathways and synergy screens for compounds that, at subinhibitory levels, enhance the activity of known antibacterials. Antisense RNA regulation of expression was devised as a genome-wide approach to identify novel antibacterial targets in *S. aureus* [69, 70]. The antisense sequences cloned behind a regulatable promoter could be turned on, leading to downregulation of the complemented gene and the identification of essential genes whose growth was inhibited when antisense RNA was strongly expressed. Where antibacterial inhibitors of specific gene products were known, antisense downregulation of those genes led to specific sensitization to the inhibitors [69]. Thus, it was recognized that such antisense strains or strains with similarly downregulatable targets could be used for screening by comparing the sensitivity of a strain with a downregulated target to that of an isogenic wild type [69, 71]. An antisense screen for inhibitors of *S. aureus* FabF (β-ketoacyl-ACP synthase II) described by workers at Merck [72] found NP inhibitors of FabF (platensimycin [73]) and FabF and FabH (β-ketoacyl-ACP synthase III) (platencin [74]). A similar antisense screen targeting gyrase B (GyrB) identified a known NP, nargenicin, which was found to be a specific inhibitor of the bacterial DNA polymerase, DnaE [75]. The concept that downregulation of one step in a pathway (such as GyrB) can lead to sensitization of other steps in the pathway (such as DnaE) was evident in the construction of the antisense array that can be used to identify antibacterial mechanism of action [59].

An NP screening strategy could be envisioned using large banks of strains, each antisense-downregulated for a single gene.

Synergy screening has been in use for >50 years, although it was not obvious in the literature until recently [53, 76–79]. It is well known that in MRSA, there are many auxiliary genes which, when inhibited or lowered in expression, will increase sensitivity to β-lactams [80, 81]. Thus, screening for agents that synergize a β-lactam against an MRSA strain should find inhibitors of these auxiliary genes, many of which would themselves be essential. A number of compounds have been disclosed by Merck, which were discovered on the basis of such synergy of a β-lactam against MRSA. In such screens, compounds are tested for their ability to inhibit MRSA in the presence of a subinhibitory concentration of a β-lactam but not (or only at much higher concentration than) in its absence. Inhibitors of signal peptidase I [78] and of a protein of unknown mechanism of action whose inhibition affects peptidoglycan synthesis specifically, SAV1754 [76], found by these screens were disclosed. Run in this way, synergy screens are hypersensitive in that they will perforce select for inhibitors which, in the absence of the β-lactam, would not show antibacterial activity on their own at the tested concentration.

2.6.3 *In Vitro* Screens for Single-Target Inhibitors

Pathway- and target-based whole-cell screens gave way to screens for inhibition of purified protein targets, almost all enzymes, chosen for their potential essentiality, and being broadly conserved among bacteria and absent from humans. Before the advent of bacterial genome sequencing, target selection was often made on the basis of microbial genetic demonstration of essentiality of the function, usually through the use of conditional mutants. As noted earlier, phenotypic screens were often based on the behavior of the mutants when grown under nonpermissive conditions. While phenotypic screens have the benefit of finding compounds that can enter cells, they require follow up to determine the actual target of any inhibitor. But the inverse is required with *in vitro* screening for enzyme inhibitors. It must not be assumed that any antibacterial activity of an enzyme inhibitor is due solely to the inhibition of that enzyme; this must be proved.

After bacterial genome sequences became available in the mid-1990s, there was a great effort toward identifying potential new targets for the discovery of novel antibacterial agents – with the hope that inhibitors of these new targets would not be cross-resistant with the classical antibacterials. Several of the large pharmaceutical companies, including Roche and SmithKline Beecham, did extensive genomic panning for novel targets. The advent of the genomic era coincided with the recognition of the rise of antibacterial resistance – of MRSA, multidrug-resistant-*M. tuberculosis* (MDRTB), vancomycin-resistant enterococci (VRE), extended-spectrum β-lactamases (ESBLs). Perhaps it was this accident of timing that turned antibacterial discovery into a search for new targets. The reasoning was that the standard antibacterials in use, the ones to which resistance was rising, had very few molecular targets and, therefore, inhibitors directed toward new genomics-revealed targets

should not be cross-resistant with the older agents. Of course, much of the preexisting resistance to these older agents was not target directed but, instead, directed toward the inhibitors themselves. However, it was indeed to be expected that new chemical classes directed at new targets would not exhibit cross-resistance. This reasoning, however, neglected the probability that, as discussed earlier, targeting of single enzymes is very likely to select for preexisting mutations in the target organism. Instead of taking significant time for resistance from exogenous sources to arise, resistance might be expected to occur overnight, during therapy. Yet genomics-/bioinformatics-based targeted discovery appears to have made up the bulk of antibacterial discovery efforts in the genomic era. This approach has not been successful in bringing novel single-targeted agents to registration – although some have reached early stages in the clinic. The reasons for this lack of output are complex, usually not disclosed and are likely due to a number of factors. These include poverty of chemical libraries for screening, reduction in screening of NPs, poor selection of targets (as discussed earlier) due to lack of essentiality *in vivo* or likelihood of rapid resistance selection and the critical problem of finding chemicals that can enter bacterial cells, especially gram-negatives, and not be effluxed from them.

2.6.4 Chemicals to Screen

2.6.4.1 Chemical Collections

A number of studies and reviews have discussed the nature of commercial and industrial chemical libraries, as to their content of interfering activities, but also to the differences between their physicochemical properties and those of antibacterial drugs. The GSK study in which their library of over 500 000 compounds was screened *in vitro* for inhibitors of 67 bacterial targets and yielded hits in only 15 of those HTS campaigns, using the criteria for a hit of chemical tractability (admittedly a subjective measure), high potency, and a preference of greater than 10-fold for inhibiting the bacterial enzyme over the homologous (or related) mammalian target [1]. The few hits led, via extensive chemical optimization, to five ''leads.'' To qualify as a lead by the GSK criteria, a hit had to have antibacterial activity and good evidence that this activity was due to inhibition of the enzyme in question. It is notable that no HTS hits were also leads. That is, even when inhibitors were found, they either had no antibacterial activity or that activity was not related to enzyme inhibition. In my experience, this appears to be a common finding in antibacterial discovery, but there are, of course, exceptions. The five GSK leads were active against enoyl-acyl carrier protein reductase (FabI), 3-ketoacyl-acyl carrier protein III (FabH), peptide deformylase (Pdf), methionyl. tRNA synthetase (MetRS), and phenylalanyl tRNA synthetase (PheRS). The FabI and MetRS lead had narrow spectra and were outlicensed to biotechs for further optimization and development (FabI to Affinium; MetRS to Replidyne (now Crestone)). No FabH or PheRS clinical candidate from GSK has been announced, and it is unclear whether the Pdf leads discussed in this study led to the current clinical candidate, now in phase II. The

GSK group ascribed the low output of their broad target-based screening program to "insufficient or improper diversity" in their library. They noted that antibacterials have different physicochemical characteristics from, for example, central nervous system (CNS) drugs. CNS drugs closely follow Lipinski's Rule of 5 (Ro5) for orally bioavailable drugs [82], whereas many antibacterial drugs do not. The Ro5 denotes physicochemical parameters that should favor oral bioavailability. The compound should have $\leq$5H-bond donors, $\leq$ 10H-bond acceptors, MW < 500, and logP $\leq$ 5. Additional analysis of the differences between antibacterials and other human health drugs have been recently published [2, 47, 49, 50].

Another study of the suitability (or rather, unsuitability) of chemical collections for screening one bacterial target [83] evaluated all hits from a screening campaign for inhibitors of AmpC β-lactamase among 70 563 compounds in the NIH Chemical Genomics Center (NCGC) library. Samples were screened in at least a seven-point dose–response series and in the presence or absence of 0.01% Triton X-100 in order to distinguish aggregating activity. Of the 70 593 compounds, there were 1274 hits. Of these, 95% were Triton-reversible aggregators, leaving 70 detergent-insensitive hits (0.1% of the total). But these consisted of β-lactams, nonreproducible activities, detergent-resistant aggregators, and promiscuous or other covalent inhibitors. There were no specific, reversible inhibitors found by standard HTS methods. However, the authors used the same library as a source for computational docking to their β-lactamase target – and found two possibly more interesting inhibitors of modest potency among 16 hits from that *in silico* analysis. Thus, false positives appeared to overwhelm any true, and especially, weak actives. The authors noted that the low output might be due to the use of a relatively small and unbiased library, and further proposed that HTS for more common targets such as G-protein-coupled receptors (GPCRs) and kinases is successful because libraries can be biased for those types of ligands.

So, HTS for inhibitors of antibacterial enzyme targets has not been very successful. As the authors of the above-mentioned two studies proposed, it is likely that Big Pharma libraries are biased in that they favor classes of inhibitors and ligands from previous campaigns to find actives on mammalian targets – and those are different in quality from the bulk of chosen bacterial screening targets. A study in 2006 of pharmacological target space [84] noted that of (529) targets that were hit by potent (IC_{50} < 100 nM) probes or drugs that obeyed Ro5, most (62.6%) were kinase or protease inhibitors, ion channel blockers, or receptor (mostly GPCR) agonists or antagonists. It seems likely that pharmaceutical chemical libraries would be similarly distributed, at least in having a lower percentage of ligands interacting with NON-kinase/protease/ion channel/receptor ligands – which are the group into which most bacterial enzyme target candidates would fall.

In addition, Big Pharma chemical libraries have many promiscuous hitters, the so-called PAIN (*p*an-*a*ssay-*in*terference) compounds [85] and detergents with antibacterial activity that hinder empirical discovery; but this is also problematic for targeted discovery. Any targeted screening hit must be tested for selectivity, to reduce potential for human toxicity, and for specificity, to demonstrate that the antibacterial activity is due solely to the inhibition of the *in vitro*-inhibited

target. Phenotypic whole-cell assays that are designed to reflect the intracellular mechanism of inhibitors can be used for secondary testing of *in vitro* screening hits that exhibit antibacterial activity. Other methods for testing specificity are reviewed in [2, 58]. As most of the present day screening seems to involve high-throughput robotic liquid handling systems, assays that have few steps after initial mixing, single-concentration dosing (generally in duplicate), are favored. They do not seem to involve validating tests for robustness by inclusion of many negative controls. In HTS, false positives and high hit rates coupled with arbitrary cutoffs for ''activity'' can obscure actual hits. One might ask whether setting up assay systems that accommodate millions of samples is worth it if the samples are mostly junk.

Given that standard chemical libraries have been problematic for antibacterial screening, how can the best use be made of them? In the study on screening for inhibitors of β-lactamase referenced earlier [83], the authors, who had found no progressable hits in their biochemical screen had, in parallel, performed docking calculations on the same library. From this approach, two compounds that had relatively weak affinity to the β-lactamase enzyme (K_is of 70 and 105 μm) but appeared to be specific were found, and both competitively inhibited the enzyme. It is possible that such a library is more useful for *in silico* rather than ''in reality'' screening. A caveat is that these findings may be limited to β-lactamase. But *in silico* screening and fragment-based design (and screening) may be a way out of the bad-library conundrum. This will be discussed briefly in subsequent text.

2.7 Natural Products

The products of fermentation of microorganisms have the benefit that the synthetic operons producing those screened compounds have been subject to evolution [86]. Those compounds have been selected for some reason, for some advantage, whether for weapons production or for signaling. An analysis of hit rates in general (nonantibacterial) screening on various sets of compounds [87] found that NPs were the most diverse set of compounds and had the highest hit rates. While diversity is no doubt important, the correlation of diversity and hit rate may obscure the role of evolution in the success of NPs as drugs and leads. For example, it seems that NPs have been selected to hit targets in the producer's local environment that have human homologs of medical interest. This is obviously the case with the HMG-CoA reductase inhibitor statin, lovastatin (mevinolin), a product of *Aspergillus terreus* and the immunosuppressants tacrolimus (FK-506) and sirolimus (rapamycin), products of Streptomyces, that have specific targets in fungi as well as in human [88, 89]. Another hypothesis explaining the utility of NPs in screening for a wide variety of protein targets is that while proteins may be varied, the number of conserved ''folds'' or architectures of proteins is relatively low and such folds may retain similar structure while the rest of the proteins are not so highly conserved [90, 91]. Thus, the local receptors for which NPs are selected may share active sites with mammalian targets that have little overall homology or similarity of function.

NPs (from microorganisms, particularly from bacteria) have evolved to target bacterial receptors, be they enzymes or structures, whether as signaling molecules or as weapons. But empirical screening of NPs will yield the common antibacterials found during the first 60 years of screening for antibacterials among Streptomycetes. So, the main goal of antibacterial NP screening is to find novelty in a way that will favor the detection of selective, targeted antibacterials. One might use assays for specific targets to search for novel types of inhibitors among NPs – but this is a seemingly inefficient use of the source (although it was one often enforced by upper management in various pharmaceutical settings). One might as well just purify NPs on the basis of UV trace and put them into standard chemical libraries. But for screening of extracts and even whole broths, the goal is to find ways of selecting for compounds not detected by the old screening methods. As noted in the phenotypic screening section, hypersensitive screens – such as reporter, antisense downregulation, and synergy screens – that are not overly target specific or can be used in parallel (as with a bank of antisense screens) can be used productively to find novelty.

Although there is no hard numeric evidence for it, in my experience, it seems that the best and most selective antibacterial antibiotics are produced by Actinomycetes and other bacteria. While fungi and plants produce many compounds with antibacterial activity, it seems that most of these are nonspecific or toxic. The penicillins and cephalosporins are an obvious exception – but it seems highly likely that the β-lactam synthetic machinery was imported into fungi from bacteria [92]. Other exceptions are fusidic acid, a steroidal antibiotic that appears to be made solely by fungi and the pleuromutilins (Table 2.2). But apart from these, all of the antibacterial antibiotic classes that are used in human or animal health are bacterial products. Fungi make a variety of antifungal-specific activities, while bacteria make the polyene class of antifungals. Perhaps bacteria make one main class of antifungal as a weapon and fungi make one main class of selective antibacterial while retaining a variety of structural classes that can affect their own domains. And this is likely related to the need to selectively kill – or signal to – other bacteria (or fungi) without overt broadly directed toxicity. There are many plant-derived NPs, including numerous flavonoids, that have antibacterial activity. Indeed, plant extracts have been used for millennia in fighting infection, but it is not clear that any of these, if purified, would have a sufficient therapeutic window for formal development. Where studied, most appear to have promiscuous activity, inhibiting a variety of enzymes, including mammalian and viral as well as bacterial. Many of these appear nontoxic, and are, in fact, ubiquitous flavoring agents, such as curcumin; but none have been developed as ethical pharmaceuticals.

It is likely that exploring novel ecological niches for unexploited organisms could lead to a higher percentage of empirically discovered novelty. In addition, methods designed to discover novel antibacterials from so-called uncultivable organisms in the environment have been described, both by cloning and expressing metagenomic DNA in likely host bacteria [93, 94] and by establishing methodology for culturing the previously uncultivable strains [95]. The hope is that these previously unstudied organisms will be found to produce previously unseen compounds – and while that

may well be true, it is unknown whether the frequency of novel compounds will be significantly higher in these new sources of organisms. Thus, if the frequency is low, then phenotypic screening methods may need to be employed.

2.8 Computational Chemistry, Virtual Screening, Structure- and Fragment-Based Drug Design (SBDD and FBDD)

Antibacterial drug design based on an understanding of the structure of a target enzyme has made great strides over the past 20 years. A recent article by Schmid covers the major outlines of this methodology [96], and other recent reviews give many specific examples [97, 98]. As Schmid indicates, most structure-based drug design (SBDD) is not done de novo, but rather is based on the structures of known ligands, inhibitors, screening hits, or *in silico* screening and/or docking programs for the initial chemical matter. With an X-ray or NMR structure in hand, various computational methods have been used to enable the development of virtual screens for inhibitors of a wide variety of bacterial enzymes. There has been some level of success reported – in that enzyme inhibitors have been found and some of them have antibacterial activity. But there seem few, if any, notable cases where such compounds have been shown to be antibacterial owing to inhibition of the enzyme of choice. For example, an interesting series of inhibitors of the DNA primase, DnaG, was identified [99] by virtual screening and follow-on testing of commercial analogs. These had antibacterial activity – but there was no evaluation of whether the antibacterial activity was due to inhibition of DnaG or DNA replication. While such compounds would, as the authors note, provide useful 3D pharmacophores for further optimization, no further work on these has been described.

In a more extensive program at Pfizer, an empirical antibacterial HTS hit that had been synthesized in a kinase-inhibitor program was found to target biotin carboxylase, an enzyme of the acetyl-CoA-carboxylase complex necessary for fatty acid synthesis [100]. The compounds had antibacterial activity due to enzyme inhibition, but the antibacterial activity was rather limited to fastidious gram-negatives and permeable *E. coli*. These workers then continued work on the biotin carboxylase target using two methods [101], a virtual screen of 5.5 million compounds based on 3D-shape homology derived from information on the initial screening hit, along with a high-concentration assay for enzyme inhibition by any identified hits, and a fragment-based approach, screening a 5200-member fragment library using the same high-concentration enzyme assay. Interestingly, the virtual screen had a hit rate 200-fold higher than an HTS screen using the same assay. From the fragment screen, 142 hits were titrated in the enzyme assay and 6 had $1C_{50}$ values below 95 μM. Several had ligand efficiencies of 0.54–0.28. Ligand efficiency is an important measure in fragment-based drug design (FBDD). It is the ratio of the free energy of binding of a ligand to a receptor divided by the number of nonhydrogen atoms in the fragment, basically a measure of the

efficiency per atom in contributing to fragment binding. So, a small fragment with high affinity may score higher than a larger, but more potent, ligand. Several hits were obtained from each method (with one hit in common), which contributed to further characterization of fragment-binding modes and led to the design of improved (relative to the initial fragment hits) inhibitors by growing some fragments and merging or morphing others. The best inhibitors had IC_{50}s of 1.29 and 2.4 μM – much higher than the original screening hit ($IC_{50} < 0.001$ μM). Their antibacterial activity was shown to be due to specific inhibition of fatty acid synthesis but their antibacterial spectrum (although at lower potency) was similar to that of the original hit. While no candidate has emerged from this work, it is an instance of fragment-based design that has demonstrated the successful generation of hits with the desired whole-cell activity, if not potency.

Several companies have approached bacterial DNA ligase as a target. It is different from human DNA ligase in that it uses nicotinamide adenine dinucleotide (NAD+) rather than ATP as a cofactor. The Bayer group [102] had the first specific compounds – a series of pyridochromanones (Figure 2.2a) identified by HTS with an *in vitro* assay for enzyme inhibition, with secondary screening by an alternate ligase assay and counterscreening to show lack of activity against the human enzyme. The pyridochromanones are potent enzyme inhibitors (IC_{50} as low as 40 nM) with good MICs (1–4 μg ml^{-1}) and bactericidal activity against *S. aureus*. The antibacterial activity was convincingly correlated with enzyme inhibition by several tests, including selection of a resistant mutant mapping in the ligase gene. AstraZeneca's DNA ligase program, described by Stokes *et al.* [103] and

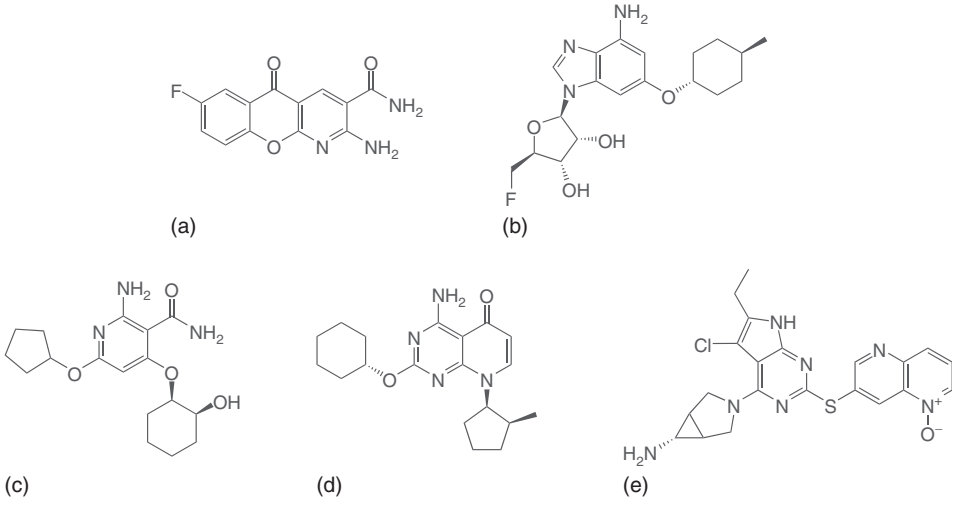

Figure 2.2 Inhibitors by screening and design. (a–d) Inhibitors of bacterial DNA ligase. (a) Bayer pyridochromanone, compound 3 [102]. (b) AstraZeneca adenosine analog, compound 4 [104]. (c) Vertex aminoalkoxypyrimidine carboxamide, compound 29 [105]. (d) Vertex aminopyridopyrimidinone, compound 33 [106]. (e) Trius pyrrolopyrimidine GyrB ParE inhibitor, compound 27 [108].

Mills *et al.* [104], was based on adenosine analogs found in HTS with a fluorescence resonance energy transfer (FRET) assay using recombinant *Haemophilus influenzae* enzyme. The initial hits had *S. aureus* and *Streptococcus pneumoniae* activity and analoging yielded improved MICs with little change in enzyme inhibition. The lead compounds were shown by a number of tests to selectively and specifically inhibit bacterial DNA ligase, and that inhibition was responsible for antibacterial activity. The lead compound (compound 4, Figure 2.2b) was bactericidal against *S. aureus* and shown to have *in vivo* efficacy in a mouse thigh infection model of *S. aureus* and a lung model of *S. pneumoniae* [104]. Vertex chemists Gu [105] and Wang *et al.* [106] have described an SBDD DNA ligase inhibitor program based on the AstraZeneca and Bayer compounds, whose binding sites are close and partially overlap. The compounds described by Gu, a series of aminoalkyl pyrimidine carboxamides (AAPCs) (Figure 2.2c), have a hybrid design that interacts with the two portions of the ligase active site defined by the AstraZeneca and Bayer compounds. Some AAPCs have potent ligase inhibitory activity but only spotty and low-potency activity against *S. aureus*, with the best antibacterial activity shown against *E. coli* strains lacking efflux and carrying a temperature-sensitive mutation in the ligase gene. Wang described a different series (Figure 2.2d) intended to improve antibacterial activity – and while a few of the compounds in this series showed moderate activity against *S. pneumoniae* and some activity against *S. aureus*, none had useful activity against wild-type *E. coli* and there were no strong leads. While resistant mutants were not described for the Vertex compounds, they were seen with the AstraZeneca and Bayer compounds; and recent work by Podos at Achillion [107] investigated the potential for resistance to the Bayer pyridochromanone, finding mutants at high frequency (4×10^{-7}) mapping to 22 different sites throughout the gene. The Achillion authors note that their work and previous work, showing that significant ligase depletion can be tolerated in *E. coli* and Mycobacteria, indicate that DNA ligase should be more thoroughly vetted as an antibacterial target. The work on DNA ligase is a good example of a likely screening target for which inhibitors were found by screening and design and validated. However, no candidates have appeared from these studies and it may be that the single-target resistance problem, as well as the probable overabundance of the target in the cell, will make the target nonviable.

A recently described program at Trius on GyrB/topoisomerase IV (ParE) inhibitors with broad-spectrum (including gram-negative) activity, combines multitargeting (GyrB and ParE, the bacterial type II topoisomerases have similar active site motifs) with SBDD, FBDD, with attention being paid at every step to cell entry [109, 108]. Initial fragment-based crystallographic screening identified a lead pyrrolopyrimidine scaffold that fit the active site pocket of GyrB and ParE from a number of bacterial strains. Optimization was carried out to obtain potent, balanced dual activity against both enzymes from a broad spectrum of bacteria and at the same time, charge distribution and physicochemical properties were modified empirically to penetrate gram-negatives, and avoid efflux. Throughout, the whole-cell mechanism of action was monitored by measurement of inhibition of DNA synthesis in preference to other macromolecular synthesis pathways. This

was important, as it was found that certain structural modifications giving high potency and broad spectrum were shown to inhibit all measured synthetic pathways equally – and were thus avoided in subsequent optimization [108]. Potent inhibitors with the desired properties were found, the best compound 27 (Figure 2.2e) having MICs against *S. aureus*, *E. coli*, *P. aeruginosa*, and *Acinetobacter baumannii* of $\leq$0.06, 2, 4, and 4 $\mu g\,ml^{-1}$. At a recent ICAAC meeting (September 2012), a new set of leads was shown to have *in vivo* efficacy and very low rates of spontaneous resistance, as would be expected from a balanced dual inhibitor. This program illustrates the necessity and worth of optimizing for many parameters simultaneously throughout iterative compound design and also emphasizes the benefit of targeting two different enzymes (as well as homologs from the desired spectrum) from the beginning of the design and optimization phase.

2.9 Conclusions

There is not much evidence that we can do successful antibacterial discovery in a completely rational way without addressing a number of critical obstacles. These include improving chemical libraries, going back to NPs, accepting that it is likely that single-enzyme inhibitors cannot be used in monotherapy and that we will have to learn how to evaluate combination therapy and, more importantly, how to develop such new combinations – which are made solely on the basis of preventing resistance – against standard (non-TB, non-Helicobacter) pathogens. We should start to look at antibacterial discovery as the same kind of problem as antiviral or anticancer therapy. Antibacterial drug discovery has the benefit over many other fields of drug discovery in that preclinical research can predict efficacy in humans with a high degree of likelihood, which is further increased if phase I pharmacokinetic data are available.

As illustrated in the historical record, perfectly good antibacterials can be arrived at empirically. And, with antibacterials, phenotypic screens can be profitably used to more selectively find compounds of interest in both NPs and chemical collections. But the era of target-directed discovery seems to have imposed a paradigm that seems rational – and yet has been very inefficient. A very recent review of the adverse effect of target-based discovery on the pharmaceutical industry as a whole (however, not touching on antibacterials) favors a paradigm shift away from the focus on drug discovery as a process and a return to a more research-based approach [110].

One take-home lesson from all these analyses is that we cannot treat drug discovery as a stepwise, modular process, but one in which all aspects must be integrated from the earliest stages. Assumptions must be continually tested and compounds must be iteratively subjected to assays of specificity, selectivity, resistance potential, toxicity, and so on, during optimization. And part of the need for integration entails the various scientists – biologists and chemists, pharmacologists, toxicologists, modelers – to participate in ongoing interaction. Communication, which

used to be a given in the days when drugs were discovered by small working groups within pharmaceutical companies, has now become international and, with outsourcing, cross-institutional. This makes multidirectional communication harder, but it should be possible to maintain it with the proliferation of electronic communication/media techniques.

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3
Impact of Microbial Natural Products on Antibacterial Drug Discovery

Gabriella Molinari

3.1
Introduction

Antimicrobial drugs or antibiotics are biologically active molecules against microorganisms. They have diverse structures and different origins: natural products, their partial synthetic derivatives, or chemically synthesized compounds. They have different modes of action, but the majority inhibits essential microbial functions such as protein synthesis, DNA replication, and cell-wall synthesis. Antibiotics are the most successful form of chemotherapy developed and applied in the past century.

The discovery of antibiotics revolutionized the history of medicine. During the golden age of antibiotics discovery, between 1940s and 1960s, almost all currently known classes of antibiotics were discovered, starting with penicillin and progressing to quinolones (synthetic), the last major new class of compounds identified and implemented in therapy in 1960. Subsequent to the marketing of quinolones, no major new class of antibiotics was introduced into the clinic until 2000, when the oxazolinidones (synthetic compounds discovered in 1978) were introduced. Afterwards, the lipopeptides (daptomicin discovered in 1986) were launched in 2003. During the 40-year discovery gap, the pharmaceutical industry lost interest in the antibiotic discovery field. Several reasons have been forwarded for this trend [1]: the discovery and development of new drugs is a long and increasingly expensive process until approval is obtained; once the new compounds finally reach the market, it is difficult to recuperate the cost invested in the development [2]; and finally, discovery efforts tended to be concentrated in chemical synthesis and the development of large combinatorial libraries for automated high-throughput screening (HTS), in the hope of reducing the time and expense involved in natural product discovery.

During the past two decades, technological advances, particularly in the area of molecular biology, genomics, combinatorial chemistry, and HTSs have provided highly efficient tools to expedite, increase, and improve drug discovery. However, despite identification of new targets and the creation and screening of

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large numbers of chemical libraries, there has been little success in identifying new leads for the development of new anti-infectives. In fact, regardless of significant advances in target discovery and validation, HTS, and genomics, there have not been new classes of antibiotics approved for human use. This failure reflects the particular challenges of antibiotic discovery [3]. An antibiotic must attack multiple target species that may readily become resistant, must be transported to and be active in multiple body compartments (in the case of intracellular parasites, it must also traverse the cell membrane), and, in addition to traversing the microbial cell membrane to access its intracellular target, it must, despite efflux and resistance mechanisms, be maintained at effective intracellular concentrations.

In parallel, the past two decades have seen a remarkable widespread increase in bacterial resistance to the major classes of antibiotics. Drug resistance is favored by inappropriate use of antibacterials in human therapy, widespread veterinary application of antibacterials, particularly in the livestock food industry as growth promoters, and the presence of resistance in microbes in the environment and food. Furthermore, alternative approaches to control microbial infections, such as stimulation of host immune responses, use of microbial viruses (bacteriophages), low-temperature treatments, and photodynamic therapy of periodontal and skin diseases, have so far not fulfilled their potential in terms of replacing antibiotic therapies.

There is an urgent need for new antibiotics. The emergence of bacterial resistance mechanisms giving class resistance, such as topoisomerase mutations compromising all fluoroquinolones [4] metallo- and beta-lactamases, compromising nearly all β-lactams [5], 16S rRNA methylases, compromising nearly all aminoglycosides [6], and upregulation of resistance, nodulation, and division (RND) efflux pumps, compromising multiple drug classes [7], leave few or no antibiotics active against multiresistant bacteria. Antimicrobial resistance is rising much faster than new antibiotics are developed and approved.

Despite these difficulties, and the exodus of the Big Pharma, antibiotic discovery from natural products continues, mostly in small companies and academic groups. However, a lack of capital to support the long process starting from the discovery to the phase I and II trials, and the very expensive phase III trials, is the major barrier that blocks translation of new leads to clinically used drugs [3].

3.2
Natural Products for Drug Discovery

The characterization of the properties that allow categorizing and prioritizing a compound as a lead for drug development continue to be a matter of further study and the concept of drug likeness provides useful guidelines in the drug discovery process [8]. This is aimed at reducing the high rate of failure in drug development, mainly pharmacokinetic failures or drug-induced toxicity [9]. The

concept of drug likeness is evolving and it is not limited by restricted rules for the classification of compounds. Properties that estimate drug likeness are helpful in the early stages of lead discovery, and can be used to sort out compounds with undesirable properties from screening libraries and to prioritize hits from primary screens [10, 11].

Biologically active small molecules possess characteristic molecular properties of mass, number of chiral centers, prevalence of aromatic rings, molecular flexibility, distribution of heavy atoms, and chemical properties. The work done by Lipinski [12] introduced ''the rule of five,'' which defines the properties relevant for the characterization of small molecules, particularly for medical use. Compounds should have a molecular mass lower than 500 Da, possess <5 hydrogen-bond donors and <10 hydrogen-bond acceptors, and have a <5 calculated octanol-water partition coefficient compatible with the ability to traverse biological membranes. Further studies summarized other characteristics for drug candidates, such as the molecular frameworks and substituents [13, 14], carried out statistical analysis of different drug databases [15], and developed the drug-like index, calculated on the basis of a comparison to known drugs [16]. However, it should be pointed out that there are drugs in the market, including many antibacterial compounds, which are exceptions to these rules, principally because of their higher molecular weight (MW) and polarity.

Drug candidates may be either natural biological products or synthetic compounds. Feher and Schmidt [17] compared data on the molecular properties, such as the number of chiral centers, rotatable bonds, unsaturations, atom types, rings, and chains, of natural and synthetic products and showed that combinatorial compounds are considerably less diverse than natural products and their derivatives. Furthermore, the diversity of combinatorial compounds is restricted to diversity space where there appears to be low diversity of natural products, thereby raising the question of the significance of combinatorial diversity in the context of biological processes (i.e., why this type of diversity was not positively selected during evolution) [18].

Natural products and derived compounds have so far been the most successful source of drug candidates. We may assume that the great advantage of the natural products is that their structural functionalities have been already prescreened by evolution. They are small molecules possessing a broad diversity in chemical space [17, 19–21] that have evolved to efficiently interact with their macromolecular targets within living organisms. Natural product structures range from very simple to extremely complex, although the vast majority have molecular masses of <1000 Da [1]. They are characterized by high chemical diversity, biochemical specificity, and high binding affinities to their specific receptors [17]. Furthermore, the evolution of natural product diversity has not only occurred within the constraints of available biosynthetic reactions and precursors but also in the context of biological utility [18]. The synthetic routes for natural product generation have coevolved with the functional requirements of their ligands [17].

3.3 Microbial Natural Products

Microbial natural products are the origin of most of the antibiotics on the market currently. Many of them have reached clinical use without any chemical modifications, which underscores the remarkable ability of microorganisms to produce drug-like molecules. Several antibiotics are made semisynthetically by chemical modification of natural products; the end compound used in therapy is thus a semisynthetic derivative. Table 3.1 summarizes the microbial natural products or derivatives that are used as antibiotics. The majority of clinically used antibiotics inhibit targets involved in essential microbial functions: protein synthesis (30S and 50S subunits of the ribosome and RNA polymerase), DNA replication (DNA gyrase), and cell-wall synthesis. Many other essential microbial functions are also present in mammalian cells, making them unsuitable targets for drug development.

At the end of the golden era of antibiotic discovery, it was thought that the reserves of new natural products were exhausted. However, taking into account that only 1% of the microbial diversity has been investigated, we can conclude that nature, and particularly the unknown microbial world, might hide a true arsenal of treasures to be discovered.

The majority of microbial natural products are secondary metabolites. These are produced and secreted by microorganisms while growing in natural communities and interacting with other organisms. It may be assumed that the genetic and metabolic costs of making secondary metabolites requires that the compound confers some advantage on the microorganism, either in defense against predators, in communication with its own and other populations as a signaling molecule [22], or in interfering with competing organisms [1]. In particular, polyketide compounds represent a major class of secondary metabolites, whose extreme chemical diversity has led to the discovery of an array of products used in chemotherapy. Nonribosomal peptides, terpenoids, and flavonoids have been also isolated from microorganisms and shown to be active in biological assays. During cultivation in the laboratory, certainly a completely different environmental situation compared that to that found in nature, microorganisms produce many compounds as a result of their secondary metabolism.

The extraction of secondary metabolites produced during growth of a library of microbial strains leads to the generation of a library of crude extracts. As a crude extract may contain more than a hundred compounds, only a fraction of these may be isolated from the mixture. Ideally, natural product screening libraries should consist of pure compounds in order to avoid confusing data resulting from mixtures. Libraries of secondary metabolites for screening may be established using as criteria for selection the chemical structure or biological activity of the compounds. However, the time and effort needed to separate a mixture into pure compounds when chemical structure is the criterion, is immense, making this strategy almost impossible. Generally, crude extracts and/or their fractions will be tested in parallel screens using a variety of assays. When biological activities are

Table 3.1 Sources of microbial natural products or derivatives used as antibiotics.

Antibiotic	Class	Target	Derivative or produced by
Amikacin[a]	Aminoglycoside	Protein synthesis	Derivative
Amoxycillin[b]	Hydroxyampicillin	Cell-wall synthesis	Derivative
Amphotericin B	Polyene macrolide	Fungal membrane	*Streptomyces nodosus*
Ampicillin[c]	Aminobenzylpenicillin	Cell-wall synthesis	Derivative
Azithromycin[d]	15 Membered azalide	Protein synthesis	Derivative
Aztreonam[e]	Monocyclic ß-lactam	Cell-wall synthesis	*Chomobacterium violaceum*
Bacitracin	Thiazolyl peptide	Peptidoglycan synthesis	*Bacillus licheniformis*
Cephalosporin	Cephem	Cell-wall synthesis	*Cephalosporium acremonium*
Chloramphenicol	Phenicol	Protein synthesis	*Streptomyces venezuelae*
Clavulanic acid	Oxa-1-penem	ß Lactamase inhibitor	*Streptomyces clavuligerus*
Clindamycin[f]	Thiooctopyranoside	Protein synthesis	*Streptomyces lincolnensis*
Dalfopristin-quinupristin[g]	Streptogramin	Protein synthesis	*Streptomyces pristinaespiralis*
Daptomycin[h]	Lipopeptide	Bacterial membrane	Derivative
Erythromycin	Macrolide	Protein synthesis	*Saccharopolyspora erythraea* *Streptomyces erythreus*
Fosfomycin	Phosphonic acid	Cell-wall synthesis	*Streptomyces fradiae*
Fusidic acid	Fusidane	Protein synthesis	*Fusidium coccineum*
Gentamycin	Aminoglycoside	Protein synthesis	*Micromonospora purpurea*
Imipenem[i]	Carbapenem	Cell-wall synthesis	Derivative
Josamycin	Macrolide	Protein synthesis	*Streptomyces narbonensis* sp. *Josamyceticus*
Kanamycin	Aminoglycoside	Protein synthesis	*Streptomyces kanamyceticus*
Methicillin[j]	Penicillin	Cell-wall synthesis	Derivative
Mupirocin	Pseudomonic acid	Protein synthesis	*Pseudomonas fluorescens*
Netilmicin[k]	Aminoglycoside	Protein synthesis	Derivative
Novobiocin	Coumarin	DNA synthesis	*Streptomyces spheroids*
Nystatin	Polyene macrolide	Fungal membrane	*Streptomyces noursei*
Penicillin	ß-Lactam	Cell-wall synthesis	*Penicillium chryseogenum*
Polymyxin	Lipopeptide	Bacterial membrane	*Bacillus polymyxa*
Rifamycin	Ansamycin	RNA transcription	*Nocardia mediterranei*
Sisomicin	Aminoglycoside	Protein synthesis	*Micromonospora inyoensis*
Spectinomycin	Aminocyclitol	Protein synthesis	*Streptomyces flavopersicus*
Streptogramin	Macrocylic peptolides	Protein synthesis	*Streptomyces diastaticus*
Streptomycin	Aminoglycoside	Protein synthesis	*Streptomyces griseus*
Teicoplanin	Lipoglycopeptide	Cell-wall synthesis	*Actinoplanes teichomyceticus*
Tetracycline	Polyketide	Protein synthesis	*Streptomyces aureofaciens*
Vancomycin	Glycopeptide	Cell-wall synthesis	*Streptomyces orientalis*

Source: Derived from and (source when not reported in the table): [a]kanamycin,[b]ampicillin, [c]penicillin, [d]erythromycin, [e]SQ-26180, [f]lincosamine, [g]pristinamycin, [h]A-21978C (*Streptomyces roseoporus*), [i]thienamycin (*Streptomyces cattleya*), [j]penicillin, [k]sisomicin.

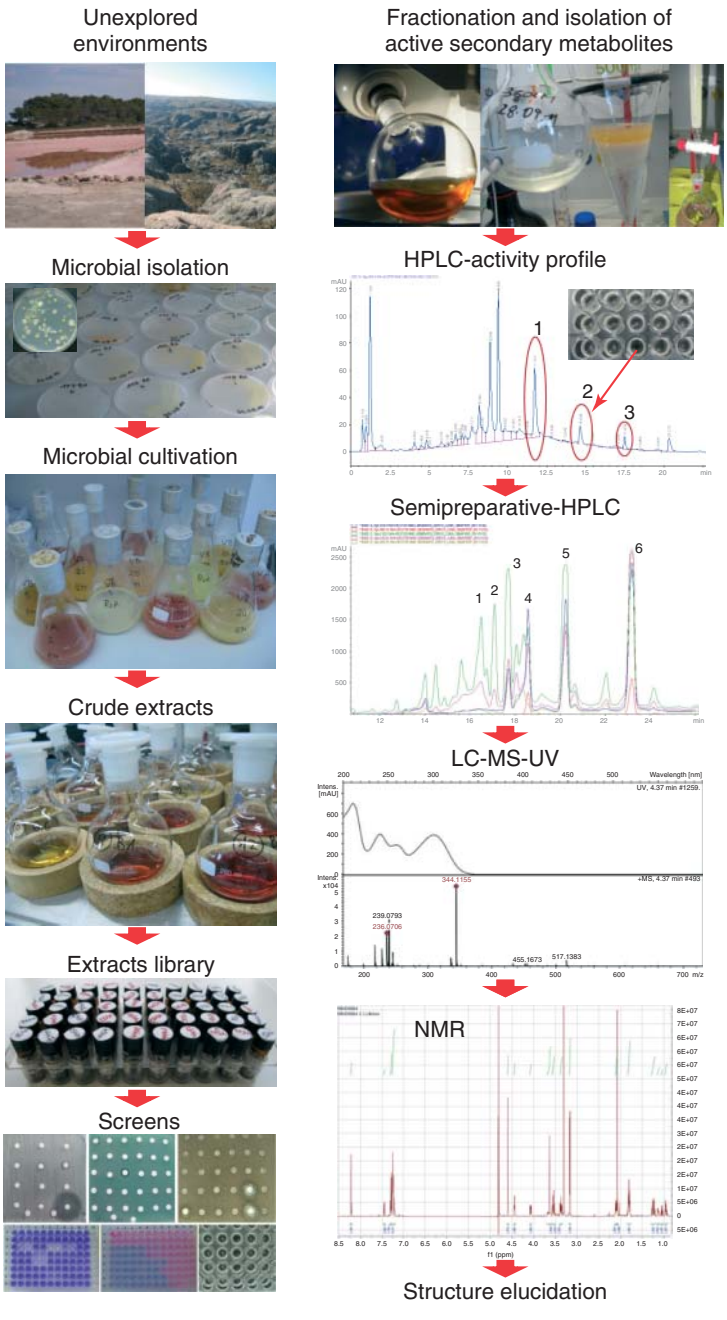

Figure 3.1 Biological and chemical steps involved at the early stage of the drug discovery process.

found (hits), a long process for the recognition, identification, and characterization of the natural product compound (lead) responsible for the biological activity is initiated (Figure 3.1). The process to develop a hit to a lead is complex, and time and resource consuming. Nevertheless, it is essential because nature contains the most rich and valuable chemical and functional diversity.

3.4 The Challenge of Finding Novel Antibiotics from New Natural Sources

The discovery of the antibacterial activity of penicillin, produced by *Penicillium notatum*, by Alexander Fleming in 1929, and the discovery of streptomycin in 1943, produced by *Streptomyces griseus*, led to an extensive screening of the products secreted mainly by soil microorganisms. Over 12 000 compounds of microbial origin with antimicrobial activity have been isolated during 80 years of drug discovery. However, only five phyla (*Actinomycetes, Bacteroidetes*, Cyanobacteria, *Firmicutes*, and Proteobacteria) include species that produce bioactive molecules that have been developed into drugs [18, 23, 24]. The majority of these are produced by the gram-positive Actinobacteria, particularly by members of the *Streptomyces* genus, as shown in Table 3.1.

The difficulty in the research based on soil bacteria lies in the high-volume screening of microorganisms required to discover novel compounds, a process rendered more difficult by the recognition of new entities in between the large number of already discovered compounds. There are many compounds produced by several species of microorganisms, as, for example, streptomycin and tetracycline that are produced by 1 and 0.4% of all soil actinomycetes, respectively. However, these genera could still hide new compounds, if we think of daptomycin, a compound discovered in 1980s, which is produced in only 0.00001% of all reported actinomycetes [25]. Recently, genome studies have revealed the presence of many cryptic biosynthetic pathways in this class of bacteria. The products of these pathways may represent the starting points for the next generation of drugs derived from natural products [26]. Furthermore, *Streptomyces* genome sequencing projects have determined that each strain contains gene clusters that encode 20 or more potential secondary metabolites. If most of the compounds that have been commercially developed as antibiotics are produced by *Streptomyces*, it is justified in assuming that this genus comprises gifted microorganisms able to produce active compounds with low toxicity. Given that not all these genes will be expressed under the same cultivation conditions, the simple expedient of systematically changing the fermentation conditions may lead to the discovery of new compounds.

The adaptability of Cyanobacteria to growth in a rainbow of environments, from terrestrial, to marine, to extreme environments such as deserts, hot springs, and the Arctic, has been attributed to their capacity to produce diverse secondary metabolites. Many bioactive compounds have been obtained from these bacteria and they are still considered a promising source for new anti-infectives, particularly now

that the increased sensitivity of analytical techniques makes easier the identification of molecules with low amounts of sample [27].

Myxobacteria are another group of gram-negative soil bacteria that have been shown to be prolific producers of a variety of bioactive secondary metabolites [28] and are being further explored by metabolomics combined with genome-mining approaches [29].

Nevertheless, the microbial world represents 90% of all biological diversity and <1% has thus far been explored [30]. Mining this microbial diversity will be the key for obtaining high compound diversity. A vast source of new natural products remains unexplored in nature [2], in the marine environments [31, 32], in salt lakes, and extreme environments, such as the deep sea [33, 34], thermal vents, volcanic sites, forests, and poorly unexplored sites.

3.5 Workflow for Drug Discovery from Microbial Natural Products

Secondary metabolite production is strongly influenced by medium components and growth conditions. The choice of the culture media for expression of secondary metabolites is difficult to make without previous knowledge of the preferred growth conditions for each microorganism. During the initial cultivation of a large number of unknown environmental strains, an optimized general medium can be used to obtain a high hit rate [18, 35]. When working with a selected group of strains, different culture media can be tested on each isolate. Alternatively, discrimination between previously tested microorganisms (dereplication of strains) may be performed using different approaches [31, 36] to tap and cultivate new diversity.

The extraction of the secondary metabolites produced during growth will lead to the generation of a library of extracts, which is stored for screening (Figure 3.1 and Figure 3.2). Parallel screens using a variety of assays allow detection of biological activities (e.g., antibacterial, antifungal, antiyeast, and antimycobacterial activities; inhibition of enzymatic processes; effects on eukaryotic cells). As a result of the primary screens, bioactive extracts are selected for further analysis. The metabolite responsible for the bioactivity must be identified from an extract that generally contains hundreds of metabolites, including known antimicrobial agents, generally present in only picogram to microgram quantities. Different chemical procedures must be used to isolate the active principles from fermentation broths and/or microbial extracts, which are complex and long procedures. The challenge is to analyze samples of mixtures that are active in a screening process, recognizing and eliminating from consideration those active substances already known, to discover new active substances.

The chemical analysis of the spectrum of metabolites present in crude extracts is difficult. The active principles contained in microbial extracts are generally isolated faster by bioactivity-guided fractionation of the crude extracts and/or prefractionated extracts by high-performance liquid chromatography (HPLC). Prefractionation of

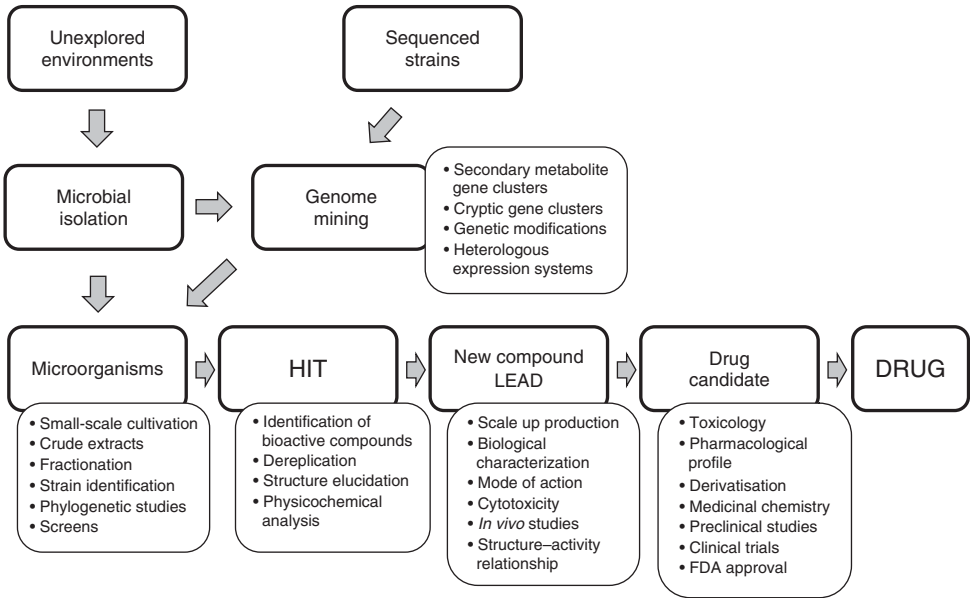

Figure 3.2 Workflow for the current drug discovery process from microbial natural products.

the extracts based on polarity is preferable, for example, using the Kupchan's partition scheme, which uses a series of two-phase mixtures in a separatory funnel to sort compounds by partition coefficient through different steps and ending with six different fractions. Other strategies for separation are applied, such as open column systems using Sephadex LH-20 and a variety of solvents that separate samples based on both size exclusion and adsorption mechanisms. Further separation is most often performed by preparative HPLC [37]. Complex profiles require the use of high-resolution equipment such as liquid chromatography (LC) coupled with either UV detection and high-throughput mass spectrometry (MS) or MS–MS, nuclear magnetic resonance (NMR), or a combination of these methods, in order to identify the active compounds more rapidly using a small amount of sample [38–40]. Comparison of MW and UV absorption data with those of compounds in databases is usually sufficient to recognize known compounds (Figure 3.1). The Dictionary of Natural Products, Chapman & Hall/CRC [41] is a comprehensive database on natural products, which includes chemical, physical, and biological properties of compounds, their systematic and common names, source, literature references, and structure diagrams. It contains approximately 68 000 entries, which can be used for the comparison of data obtained from biologically active natural products being characterized. Some research groups working in drug discovery are also building their own databases, which, unfortunately, are not always available to other groups. Natural product profiling and dereplication, the process of differentiating known secondary metabolites from the new ones, are powerful tools for speeding up the discovery process and avoiding the time and resources spent on repetitions. To

effectively shorten compound identification, generally requiring multimilligram samples, Lang *et al.* [42] developed an HPLC bioactivity profiling/microtiter plate technique in conjunction with capillary probe NMR instrumentation and access to appropriate databases, which requires only a single, submilligram sample. The dereplication of crude extracts was demonstrated with fungal or bacterial extracts containing known compounds and the identification steps were carried out on microgram quantities of extract, confirming the discriminating power of ^{1}H NMR spectroscopy as a dereplication tool [42]. Once active compounds have been isolated and purified, their structure must be elucidated by NMR analysis combined with high-resolution MS, which provide very precise mass data. The physicochemical characterization of a new molecule might be completed by UV, IR, and optical rotation analysis.

A new agent might be first recognized and later characterized from samples obtained from small-scale fermentation of the producer microorganism. However, once a compound is selected as a lead for further development, large-scale fermentation will follow. Optimization of the production process with the aim of obtaining higher yields is fundamental. However, the risk that an optimal culture medium, which allows obtaining higher yields of the desired compound, does not facilitate the chemical purification process exists. On the contrary, many culture media give very ''dirty'' extracts that, although showing higher biological activities, are difficult, time and reagent consuming to process. Most of the time, it is worth and/or necessary to invest further efforts in microbiological studies to increase the efficiency of production of the new compound and to find the appropriate culture medium to facilitate the chemical purification process. The factors that induce the production of active metabolites are poorly understood. Furthermore, it is a common experience that when regrowing the microorganism under the same conditions under which the initial crude extract was obtained, less or no production of the desired metabolite is obtained. The reasons for these problems are poorly understood and clearly there are a variety of factors influencing secondary metabolite production in nature, which are not replicated in the laboratory.

Taxonomic identification of the environmental microorganism producing a new compound is required before large-scale fermentations, both for safety considerations (it should not belong to phyla known to contain pathogens or, if so, must be fermented under high-security conditions) and to guide selection of the culture media and conditions. Taxonomic identification of isolates is performed by 16S rDNA sequence analysis. Microbial DNA is extracted and the 16S rRNA gene is polymerase chain reaction (PCR) amplified using universal 16S rDNA primers [43]. The 16S rDNA sequences are compared with reference sequences in databases for the identification of the species or genus. Phylogenetic studies might follow for the determination of the phylogenetic relationship of the isolate to validated microorganisms and determine its affiliation. When the producer microorganism shows sequence similarity with a validated and deposited strain, DNA hybridization studies between both strains will determinate if the new environmental-isolated strain could be considered a new species. Further metabolic and phylogenetic studies are necessary for the description of a new microbiological species.

Large-scale fermentations are necessary for the production of enough material for chemical and biological characterization, including (i) structure elucidation and chemical characterization, (ii) biological characterization, (iii) elucidation of the mode of action, (iv) cytotoxicity studies, and (v) pharmacological profiling. During large-scale fermentation, product recovery and purification, bioassays need to be performed at each level for the verification of the stability and activity of the new compound. Moreover, synthesis and testing of a series of derivatives might provide insights into a structure–activity relationship and pharmacokinetic aspects. Finally, drug leads go to toxicological studies and *in vivo* studies and, if successful, become drug candidates (Figure 3.2).

The total synthesis of a new active compound, once its chemical structure has been elucidated, may be thought to be the strategy to follow. However, the total syntheses of complex natural products are generally academic exercises that will rarely be commercially feasible. Nevertheless, the knowledge obtained from total synthesis will contribute to the understanding of the new structure–activity relationship and provide the possibility of producing derivatives or more potent analogs.

To facilitate and speed up the frame time for natural product drug discovery, several new analytical technologies need to be further developed and implemented: high-throughput small molecule separation, analysis technology, MS coupled with searchable chemical databases that include mass spectral fingerprints to rapidly analyze natural product crude extracts and recognize new compounds, while avoiding known ones.

3.6
Antimicrobial Activities: Targets for Screens

Libraries of microbial crude extracts, their fractions, or pure natural product compounds are tested in phenotypic screenings to identify biological activities. When searching for antimicrobial activities, microorganisms and/or microbial targets are the starting point of the screens.

The use of whole bacterial target cells in the screens for detection of antibacterial activities is very important. Results obtained with whole bacterial cells give direct information about effectiveness. A new compound must not only show activity, it must have the ability to penetrate bacterial cells to reach its target. Particularly, in the case of gram-negative bacteria, a complex outer membrane and a set of efflux pumps need to be overcome before the target can be reached; in the case of gram-positive bacteria, a thick cell-wall layer needs to be trespassed. The setup of ''intelligent'' screens helps avoid the rediscovering of known compounds as, for example, the use of multiresistant ''superbugs'' and/or genetically modified cells that produce specific responses on inhibition of a selected function [44, 45]. In particular, the use of a group of target strains with different patterns of resistance to the different classes of antibiotics in use allows the rapid selection of extracts whose activities cannot be accounted for by antibiotics currently in use. Furthermore,

some extracts, while not active against sensitive target strains, are active against resistant strains, leading to the discovery of new and specific active compounds that might have new modes of action.

HTSs use robotics for liquid handling, instruments with sensitive detectors, control software, and data processing, allowing to quickly conduct millions of tests. Hits are the results of these screens, which provide starting points for investigating the interaction between a molecule and a target in a particular biochemical process. HTSs are applied when large compound libraries are available. Generally, in natural product research, the compounds are variations of the natural molecule obtained by combinatorial chemistry. However, large compound libraries formed with many different natural products are an almost impossible task to obtain and maintain in storage for HTS using multiple targets. Moreover, HTS campaign speed does not match the slow process of natural product acquisition [1]. Libraries from crude extracts, and not purified compounds, are most likely to be produced from environmental microorganisms. The difficulties with these libraries and HTSs are that the concentration levels of compounds might not be enough for detection of activity by HTS and/or the possible presence of compounds that inhibit the test. Moreover, many HTS assays are based on fluorescence reactions and extracts may contain compounds that have high fluorescence or that otherwise affect the readout of the assays. Complementary to HTS, which tests large libraries of compounds, is compound fragment-based drug discovery (FBDD), which tests only several thousands to 20 000 of small (<300 Da) highly soluble molecules [46]. This screen provides hits with higher quality chemical properties and aims to lower the number of false positives. However, its success depends first on the fragment library design.

The influence of genomics in drug discovery will be mentioned also later when discussing the detection of novel or cryptic biosynthetic gene clusters in microorganisms by genome mining. On the other hand, the influence of the developments in genomic technologies has also implications in the identification of new targets for drug discovery from microbial pathogens, the target microorganisms. Essential genes could be identified in the genome of pathogens; their gene products, usually enzymes or receptors, participate in a process that could be the basis for the development of an assay to be used in HTS of compound libraries, to allow the identification of molecules interfering with the process. The active compounds are leads for further development, which can be modified by chemical synthesis to improve target affinity and their pharmacological characteristics. They also need to be further characterized and validated in whole-bacterial cell assays and, in parallel, tested in animal model of infections. It is a challenge for a compound active in a target-based assay, performed *in vitro*, to keep its activity when confronted with the target bacteria. In this area of research, although new targets have been identified and several leads have been selected, no compound has yet passed clinical trials.

The identification of new targets might be a powerful approach, which might lead to the discovery of new classes of antibacterial compounds. However, the recognition and binding between a target and a compound does not correlate with antibacterial activity. This is the major limitation in the use of HTS for antibacterial drug discovery. Nevertheless, compounds active against an isolated

target and not against the bacterial whole target might be used for the development of synthetic derivatives, which could overcome the deficiencies of the original compound. Impermeability or toxicity problems could often be solved by chemical modification. Moreover, they should be kept apart waiting for the development of alternative approaches using delivery systems. Nanoparticle carriers can mediate the encounter between compound and molecular target and thereby facilitate the interaction. These new approaches have been implemented in the anticancer field, and they are expected to be very promising developments also for anti-infectives [47–50].

3.7
Natural Products: A Continuing Source for Inspiration

The review by Newman and Cragg [51] analyzed the sources of new drugs from 1981 to 2010, and shows that almost 50% of new drugs launched during this period have a natural product origin. Furthermore, Mishra and Tiwari [52] described the natural products that have undergone clinical evaluation or registration from 2005 to 2010, indicating that natural compounds are still a viable source of new drugs. Currently, natural products discovered in screenings (leads) are the starting point for medicinal chemistry programs aimed at enhancing their biological profiles [26]. In medicinal chemistry, chemical principles are applied to modify natural compounds to design effective therapeutic agents; a drug is developed from a lead. Organic chemistry knowledge must be coupled with a broad understanding of the molecular biology related to cellular drug targets. The discipline also works in the revalidation of known compounds by chemical modifications. Many active natural compounds were withdrawn from further characterization for different reasons such as solubility, stability, low production yields, and so on. These compounds, if possessing powerful activities, could be refished and developed using advanced technologies now available and in development. Furthermore, antibiotics are modified with the aim of improving their properties and bypassing the mechanism of resistance. Compounds that are not able to reach their target by impermeability problems can be linked to a cell-permeable molecule or attached to delivery systems as nanoparticles, as mentioned earlier.

The case of daptomycin, a derivative of the compound produced by *Streptomyces roseosporus*, discovered in 1980, was approved in 2003 and is an example of new application of a known antibiotic. This lipopeptide antibiotic has a MW of 1620, which is larger than Lipinski's ideal value of 500, and is used for complicated infections caused by gram-positive organisms. Other members of this family of acidic cyclic lipopeptide antibiotics are produced by different *Streptomyces* species [53].

Radezolid, an oxazolidinone (synthetic) antibiotic currently undergoing clinical evaluation, is an example of redesigning of a drug and was obtained by exploiting the knowledge acquired by the atomic structure of the 50S ribosomal subunit. It shows an improved safety profile and higher activity than other compounds of the

same class. However, linezolid, launched in the 2000s, is so far the only FDA-approved oxazolidinone in use active against vancomycin-resistant enterococci and methicillin-resistant staphylococci [54].

Tigecycline, the first glycylcycline launched in 2005, is a semisynthetic derivative of the tetracycline, produced by actinomycetes, and another example of structural modifications of known and old antimicrobial compounds aimed at overcoming resistance and improving the pharmacological properties. Tigecycline evades acquired efflux and target-mediated resistance to tetracyclines in gram-positive and gram-negative bacteria, but not chromosomal efflux in *Pseudomonas* and *Proteeae* [55].

Platensimycin and platencin, produced by *Streptomyces platensis*, are potent antibiotics active against gram-positive pathogens, including antibiotic-resistant strains and *Mycobacterium tuberculosis*. These natural products were discovered using a target-based whole-cell antisense differential sensitivity assay, as inhibitors of type II fatty acid biosynthesis, a type of biosynthesis that is not present in humans [56]. Currently, there are no bacterial lipid metabolism inhibitors used as antibiotics. Further work is required to investigate the selectivity of these compounds, although this seems to be a promising approach for finding a new class of antibiotics inhibiting a previously untargeted bacterial pathway.

Examples of synthetic molecules that mimic natural products are the peptides, particularly cationic peptides inspired by *inter alia* natural host immunity compounds, bacteriocins and defensins [57]. This approach is becoming a very interesting and promising area of research.

In biosynthetic medicinal chemistry, the producer microorganism is genetically engineered to modify a step in the biosynthesis of the active compound, thereby leading to the synthesis of a more suitable compound. The manipulation of the biosynthetic pathway was a successful strategy applied on *Actiosynnema pretiosum* for the production of macbecin, an Hsp90 inhibitor antitumor compound [26, 58]. Moreover, tandem approaches could combine the manipulation of the biosynthetic pathway followed with semisynthetic processes to obtain better and more active compounds, already starting the beneficial alterations from the production step.

3.8 Genome Mining in Natural Product Discovery

The massive availability of genomic information opens new perspectives in natural products discovery, which might undergo very exciting developments in the post-genomic era. The involvement of genomic approaches in the search for and identification of new targets has already been mentioned. Until now, this approach has not contributed as expected to antibiotic discovery and failed to bring new compounds to the market. However, with the high speed in development coupled with the reduced cost in sequencing technologies, another branch of genomic approach has started to be implemented in the discovery of new microbial natural

products. New technologies offer the possibility of rapid and cost-efficient sequencing of entire bacterial genomes to scan for unique gene clusters for secondary metabolism. Moreover, classical approaches, such as characterizing active products from microbial metabolism, could be supported by genetic approaches to identify the genes involved in secondary metabolism. Thus, there are several areas where genomics could be applied to improve drug discovery development.

Genetic knowledge is fundamental to biosynthetic medicinal chemistry, where genetic modifications could facilitate the production of "better" leads. Metabolic engineering aims also to manipulate the regulatory systems that control secondary metabolite production for the rational improvement of fermentation yields [59, 60].

Furthermore, genome scanning for biosynthetic pathways allows selecting potential producers from sequenced strains, and the selected strains can then be grown for metabolite analysis [61]. Moreover, cryptic gene clusters may be activated or expressed in heterologous hosts [62–64]. Genome searches are aimed at identifying new polyketide synthase (PKS) and nonribosomal peptide synthase (NRPS) genes, based on homology to known PKS and NRPS motifs genes [65]. The stambomycins, 51-membered macrolides produced by *Streptomyces ambofaciens*, were discovered by genome mining of a strain already known for its capacity to produce other active compounds, although the new compounds were not produced under laboratory growth conditions [66]. Genome scanning techniques are being developed to reduce to a minimum the amount of sequencing and to scan genomes of bacteria for their secondary metabolite biosynthetic genes. This direct genome analysis led to the discovery of novel secondary metabolites from *Amycolatopsis orientalis*, a strain deposited as a vancomycin producer [67], and from *Micromonospora echinospora* ssp. *chalisensis* [68].

Biosynthetic pathways that lead to active secondary metabolites are organized in modular gene clusters, which can be expressed in heterologous organisms for the production of the desired new compound [69]. Novel heterologous expression systems are being used and developed to address the problems encountered with difficult microorganisms or when the biosynthetic potential of noncultivable bacteria needs to be explored [70]. However, the further development of valid heterologous systems for expressing novel biosynthetic genes and the establishment of tools to realize the potential of easy genome sequencing in combination with bioinformatics still represents a challenge.

3.9 Conclusions

In the continuous search for new and better drugs, it is difficult to define a strategy as being the more straightforward. Natural products, after 30 years of marginalization, are receiving new interest and being favored over other strategies that have failed to produce new drugs. Natural products can still be identified on the basis of cultivation-dependent approaches from unexplored sources. Classical microbiological approaches can be extended by the application of genome-driven

approaches. Advanced technologies can contribute to the development of ''better'' compounds originating from natural products. Interdisciplinary teams combining chemical, biological, genetics, and informatics expertise contribute to shorten the timeframe required for drug discovery processes. Moreover, emerging technologies coupled with new high-resolution and powerful instrumentation will help speed up this process, thereby tackling the challenge represented by the discovery and subsequent exploitation of the treasure concealed in the nature.

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4
Antibiotics and Resistance: A Fatal Attraction

Giuseppe Gallo and Anna Maria Puglia

4.1
To Be or Not to Be Resistant: Why and How Antibiotic Resistance Mechanisms Develop and Spread among Bacteria

The continual battle between humans and the multitude of microorganisms that cause infections and diseases has caused significant morbidity and mortality throughout history. The situation significantly improved when penicillin and other classes of antibiotics were discovered and used to treat infectious diseases. However, almost as soon as antibacterial drugs were introduced in clinics, bacterial resistance spread [1, 2].

Antibiotic resistance can be defined taking into account the pharmacokinetic and pharmacodynamic criteria to determine values above which a therapeutically useful concentration is difficult to obtain. If the minimal inhibitory concentration (MIC) for a bacterium is above those concentration values, a risk exists that the infection cannot be successfully treated. Therefore, the microorganisms are classified as resistant when their MICs are above a predefined threshold.

Bacterial resistance is a concern for several reasons. From a medical, social, and economical viewpoint, resistant bacteria, becoming commonplace in healthcare institutions, often result in treatment failure and this implies an added burden on healthcare costs [3]. In addition, resistant bacteria may also spread and become broader infection-control problems, not only within healthcare institutions but in communities as well [4, 5]. From a biological and microbiological viewpoint, antibacterial drug resistance is a fascinating aspect of molecular evolution and selection of fine mechanisms that allow survival under unfavorable circumstances. In particular, under the selective pressure of antibiotics, bacteria evolve and spread resistance mechanisms that become common to pathogenic and nonpathogenic strains. To fully understand the evolution of resistance, the maintenance of resistance genes within microbial populations and the spread of these genes between species and genera, the concept of ''resistome'' was introduced [6]. The resistome includes the totality of those genetic elements whose function is to counteract toxic effects of antibiotic drugs. Furthermore, the resistome also comprises the collection of genes, called *protoresistance genes*, which have the

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potential to evolve into resistance elements [7]. Many resistance genes have been isolated from clinically relevant strains and from the vast reservoir of environmental nonpathogenic organisms.

Antibiotic-producing environmental bacteria most probably are the original source of many resistance enzymes, reflecting a continuous evolutionary pressure where antibiotic biosynthesis and resistance coevolve [7, 8]. In fact, in soil environments, evolutionary pressure promotes the development and spread of resistance genes among pathogenic and nonpathogenic bacterial genera. This hypothesis is supported by the presence of resistance elements in antibiotic-producing bacteria that have orthologs in clinical isolates [9–12]. Anyway, antibiotic-producing bacteria could not be the sole source of resistance genes. In fact, bacterial genomes contain an unexpected number of genes encoding putative resistance proteins [13–15], which could have originated through amplification and random mutation of genes not originally involved in antibiotic resistance, [16]. Primary sequence analysis of resistance proteins, determination of their molecular mechanisms, and three-dimensional structures revealed homologies to known metabolic and signaling enzymes with no antibiotic-resistance activity [7]. Therefore, it is possible that resistance genes originally derived from elements having other metabolic functions, similar to housekeeping genes encoding enzymes with modest and fortuitous resistance properties, evolved into resistance enzymes as a result of selective pressure of antibiotic exposure. The fact that resistance genes are so widespread in the environment and that even resistance to synthetic antibiotics can be readily selected reveals the plastic nature of the link between molecular evolution and resistome, whose origins may predate the actual antibiotic era [17].

4.1.1 Horizontal and Vertical Transmission of Resistance Genes

Despite the wide range of chemical complexity of antibiotics, there are five major modes of action (interference with cell-wall synthesis, inhibition of protein synthesis, interference with nucleic acid synthesis, inhibition of cofactor biosynthetic pathways and membrane pore formation) and bacteria may manifest resistance to antibacterial drugs through a restricted range of molecular events (Table 4.1; Figure 4.1). In particular, some bacterial species are considered intrinsically resistant to a class of antibiotics because the drug cannot reach its cellular target or because the drug is not able to recognize its target which possesses the same function but a different structure. As a case of intrinsic genetic arrangement conferring resistance to β-lactams, the *Pseudomonas aeruginosa* resistome [18] is described in this chapter (Section 4.5.1). On the other hand, susceptible bacteria may become resistant to a class of antibiotics through two types of genetic events:

1) random spontaneous mutation;
2) acquisition of the genetic information encoding resistance from other bacteria.

Table 4.1 Cellular targeting of antibiotic compounds and resistance strategies.

Mechanism of action	Antibiotic target	Antibiotic class (examples)	Mode of resistance	Resistance gene examples (products)
Interference with cell-wall synthesis	Transpeptidases	β-Lactams (penicillins, cephalosporins, carbapenems, monobactams)	Hydrolysis Efflux Altered target	*ampC, blaZ* (β-lactamases) *oprM* *mecA* (low-affinity PBP2a)
	D-Ala-D-Ala	Glycopeptides (vancomycin, teicoplanin)	Altered target	*vanRS* (two-component system) *vanHAX* (dehydrogenase, didpeptidase, ligase) *vanY* (peptidase)
	Lipid II	Lantibiotics (nisin)	Hydrolysis Efflux Binding	*nsr* (protease) *nisEFG* (transporter system) *nisI* (lipopeptide)
	MurA	Epoxide (fosfomycin)	Hydrolysis	*fosA, fosX*
Protein synthesis inhibition	50S ribosomal subunit	Macrolides (erythromycin, chloramphenicol, linezolid, tylosin)	Hydrolysis Glycosylation Acetylation Phosphorylation Efflux Altered target	*ereA, ereB* (macrolide esterase) *mtg, oleI,* and *oleD* (glycosyl-transferases) *cat* (acetyltransferase) *cpt, mph* *mtd(A)* *ermE* (23S rRNA methyltransferase)
		Lincosamides (lincomycin, clindamycin)	Nucleotidylation Efflux	*linA, linB* *mtd(A)*

(*continued overleaf*)

Table 4.1 (*Continued*)

Mechanism of action	Antibiotic target	Antibiotic class (examples)	Mode of resistance	Resistance gene examples (products)
	30S ribosomal subunit	Aminoglycosides (apramycin, streptomycin, spectinomycin, gentamycin)	Phosphorylation Acetylation Nucleotidylation Efflux Altered target	*aphI*, *neo*, and *km* (phosphotranferases) *aac*(3)IV (apramycin acetyltransferase) *aadA* (streptomycin adenyltransferase) *norM* (transporter) *grmM*, *kamC*, and *kan* (16S rRNA methyltransferases)
		Tetracyclines (chloramphenicol, tigecycline)	Monooxygenation Efflux Acetylation Altered target	*tetX* (monohydroxylase) *tetA*, *mtd(A)* *cat* *tetO*, *tetM*, and *otrA* (ribosome protection proteins)
Nucleic acid synthesis inhibition	DNA synthesis enzymes	Quinolones and fluoroquinolones (ciprofoxacin)	Acetylation Efflux Altered target	*aac6′*-ib *norA*, *norM*, *acrAB* *gyrA*, *parC*
		Aminocoumarins (novobiocyn)	Efflux Altered target	*simX* *gyrB*

	RNA synthesis enzymes	Rifampicin (rifamycin, rifampin)	ADP-ribosylation Monooxygenation Efflux Altered target	*arr* (ADP-ribosyltransferase) *acrAB* *rpoB*
Metabolic pathway inhibition	Folic acid synthesis	Sulfonamides (sulfamethoxazole)	Efflux Altered target	*acrAB* *sul1, sul2* (dihydropteroate synthetase)
Disruption of bacterial membrane	Cell membrane	Polymyxin (colistin)	Efflux Altered target	*acrAB* *pmrAB* (Two component system), *pmrEF* (UDP-glucose dehydrogenase, glycosyltransferase)
		Lipopeptides (daptomycin)	Altered target	*cls* (cardiolipin synthase)

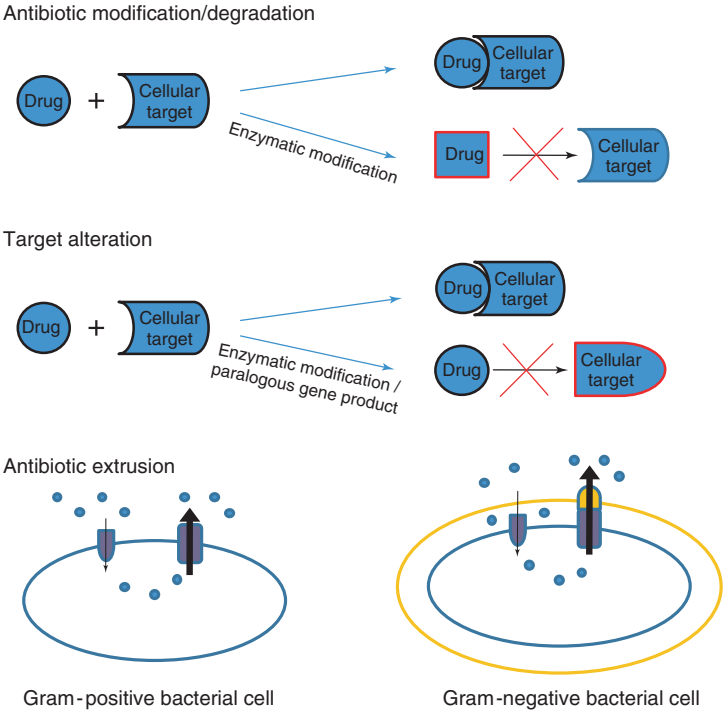

Figure 4.1 Schematic representation of major resistance strategies. Antibiotics can be destroyed or chemically modified by enzymes produced by resistant bacteria. On the other hand, antibiotic targets can be altered to ward off antibiotic recognition. When antibiotic target is located inside cells, antibiotic–target interaction can be prevented by pumping the antibiotic out from cells through efflux pumps to keep low the intracellular drug concentrations.

In susceptible bacteria that acquire resistance by spontaneous mutations resistance may be conferred by:

1) modification or loss of the target with which the antibiotic interacts (e.g., change in penicillin-binding protein 2b in *Pneumococci*, which results in penicillin resistance);
2) upregulation of enzymes that inactivate the antimicrobial agent (e.g., β-lactamases that destroy the β-lactame antibiotics) or that modify the antibiotic target (e.g., ribosomal methylase in *Staphylococci* preventing erythromycin binding);
3) downregulation or inactivation of the outer membrane protein channel required by the drug for cell entry (e.g., OmpF in *Escherichia coli*);
4) upregulation of pumps that expel the drug from the cell (e.g., efflux of fluoroquinolones in *Staphylococcus aureus*).

In all these cases, strains of bacteria carrying chromosomal mutations conferring resistance survive and grow under the selective pressure of antibiotic use, which

instead kills the susceptible strains and promotes spreading of resistant genotypes. This kind of selection is named *vertical evolution* because resistance-associated genetic elements are transmitted from cell to cell through cell duplication [2, 19].

Bacteria also develop resistance through the acquisition of new genetic material from resistant organisms. This kind of selection is termed *horizontal evolution*, and may occur in an intra- or interspecific way or even among different genera and may be facilitated by transposable elements such as transposons, which contain resistance genes [19]. Genetic exchange mechanisms include events such as conjugation, transduction, and transformation [2, 19]. During conjugation, a gram-negative bacterium transfers a plasmid carrying resistance genes to a recipient bacterium through a mating bridge, which joins the two bacteria. In gram-positive bacteria, exchange of DNA by conjugation is usually triggered by sex pheromones, which facilitate the clumping of donor and recipient cells. During transduction, resistance genes are transferred via bacteriophage. Finally, the so-called competent bacteria may acquire and incorporate resistance genes from other bacteria that have released their DNA into the environment after cell lysis, by transformation [2, 19]. Through genetic exchange mechanisms, many bacteria become resistant to multiple classes of antibacterial agents, and these multidrug-resistant (MDR) bacteria (e.g., resistant to at least three antibacterial drug classes) are a serious problem, particularly in hospitals and other healthcare institutions where they occur very commonly.

Mutation, genetic exchange, and selection cause quick adaption to the introduction of antibiotic drugs into their environment. In rare cases, a single mutation may be sufficient to confer high-level resistance on an organism (e.g., high-level rifampicin resistance in *S. aureus* or high-level fluoroquinolone resistance in *Campylobacter jejuni*). In most cases, a single event, even if in a key bacterial gene, may only slightly reduce the susceptibility to an antibiotic, but it may be just enough to allow its initial survival until it acquires additional mutations or additional genetic information resulting in a high resistance level [2]. As an example of a gene acquisition/mutation series conferring resistance, the organism may first acquire gene-encoding enzymes that destroy the antibiotic, thus reducing its overall concentration; then, bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell. Finally, bacteria may acquire several genes or accumulate mutations that produce a product not recognized by the antibiotic agent, or in the case of gram-negative bacteria, may acquire mutations that limit access to the intracellular target via downregulation of porin genes. As a real case of gene acquisition series, resistance mechanisms of *S. aureus* [20] are described in this chapter (Section 4.5.2).

4.2 Bacterial Resistance to Antibiotics by Enzymatic Degradation or Modification

Antibiotic resistance coevolved with biosynthesis as a means of bacterial self-immunity strategies for the production of toxic secondary (e.g., dispensable for

bacterial growth, at least under laboratory conditions) metabolites in antibiotic-producing bacteria [7, 8]. This coevolution strategy could have independently evolved *de novo* in nonproducing organisms or could be imported via horizontal gene transfer. The genes for resistance, stably integrated into the genome under selective pressure, reflect prior exposure during the evolution of the species. This idea is also consistent with the hypothesis that naturally produced antibiotics do not exert antibiotic activity at the concentrations present in the environment, but rather they play a role as signaling molecules [7, 21] and resistance elements could have evolved as receptors or mediators of such signaling molecules. Furthermore, antibiotic inactivation mechanisms share many similarities with well-characterized enzymatic reactions involved in primary metabolism [7]. Enzymes that confer resistance by destroying or modifying antibiotics utilize a set of chemical strategies that can be functionally grouped into hydrolysis, group transfer, and redox mechanisms (Table 4.2) [7, 12].

4.2.1 Antibiotic Resistance by Hydrolytic Enzymes

The integrity of chemical structure is essential for antibiotic activity. Thus, several kinds of enzymes confer resistance by targeting and cleaving chemical bonds that are hydrolysis prone. The best-known examples are the amidases that cleave the β-lactam ring of the penicillin and cephalosporin classes of drugs. Other examples include esterases (macrolide resistance) and ring-opening epoxidases (fosfomycin resistance). These enzymes require water for catalysis and are excreted by bacteria, so that they intercept the antibiotics before they come into contact with their bacterial target [7, 12].

Table 4.2 Antibiotic resistance by enzymatic modification.

Strategy	Type	Example enzymes	Targeted antibiotic classes
Hydrolysis		BlaZ	β-Lactams
		EreA, EreB	Macrolides
		FosA, FosX	Epoxides
Group transfer	Phosphoryl	APH(3′)	Aminoglycoside
		MPH	Macrolide
	Acyl	CAT	Chloramphenicol
		AAC(6′)	Aminoglycoside
	Nucleotidyl	ANT(2′)	Aminoglycoside
		LinA, LinB	Lincosamide
	ADP-ribosyl	ARR	Rifamycin
	Glycosyl	Mtg	Macrolide
		Not characterized	Rifamycin
Redox	Oxidation	TetX	Tetracycline
		Iri	Rifamycin

4.2.1.1 β-Lactamases

The first antibiotic-resistance strategy reported in the literature is the production of the β-lactamase penicillinase by pathogenic *E. coli* [1]. There are two main classes of β-lactamases based on the molecular mechanism of hydrolysis of the β-lactam ring: (i) Ser-β-lactamases, such as BlaZ, that work through the action of a Ser nucleophile active site and (ii) metallolactamases that activate water through a Zn^{2+} center [7, 12]. β-Lactams bind peptidoglycan transpeptidase preventing cross-linking, eventually compromising cell-wall integrity (Figure 4.2). Indeed, there is a similarity between peptidoglycan transpeptidases and Ser-β-lactamases concerning molecular mechanism of action and three-dimensional structure. Therefore, it has been speculated that peptidoglycan transpeptidases and BlaZ-like lactamases are evolutionarily linked [7, 12, 22]. The *blaZ* gene is present in plasmids and its expression is under the control of two regulatory genes, *blaI* and *blaR1*. The product of the latter gene is a sensor-transducer, which, in the presence of penicillin, initiates a cascade of events that leads to enhanced penicillinase expression. Metallo-β-lactamases are members of the Zn-dependent hydrolase family and are a significant cause of resistance to carbapenems in gram-negative bacteria [7, 12, 23].

4.2.1.2 Macrolide Esterases

The macrolide antibiotics, such as erythromycin, block the peptide exit tunnel of the large subunit of the ribosome and, as a result, interfere with protein synthesis. Macrolides are cyclized by a thioesterase responsible for the ring closure step that generates 6-deoxyerythronolide B (for the 15-member erythromycin) macrocyle [12, 24]. Therefore, this key bond is targeted by macrolide-resistance enzymes operating in reverse ring-opening mode. Two erythromycin esterases, encoded by *ereA* [25] and *ereB* [26] genes and first isolated from two different *E. coli* strains, share 43% similarity [12]. Both proteins result in very high levels of resistance in *E. coli* [27]. The presence of these genes on mobile genetic elements [28] implies their ability to become widespread in the microbial community and the presence of esterases has been confirmed in at least one clinical isolate of *S. aureus* [29] and in environmental isolates of *Pseudomonas* sp. [30].

4.2.1.3 Epoxidases

The epoxide antibiotic fosfomycin covalently modifies the enzyme MurA, an essential protein required for the synthesis of *N*-acetylmuramic acid, one of the sugarbuilding blocks of cell-wall peptidoglycan. Enzymatic resistance to this antibiotic occurs through destruction of the reactive epoxide by ring opening [12]. The enzyme FosX, whose gene was first isolated from the nonpathogenic soil bacterium *Mesorhizobium loti* [31] and FosA, a metalloenzyme found in gram-negative bacteria [32], catalyze epoxide ring opening through water- and glutathione-dependent reactions, respectively [12]. Both enzymes require a catalytically important divalent metal cation (Mn^{2+}) [12].

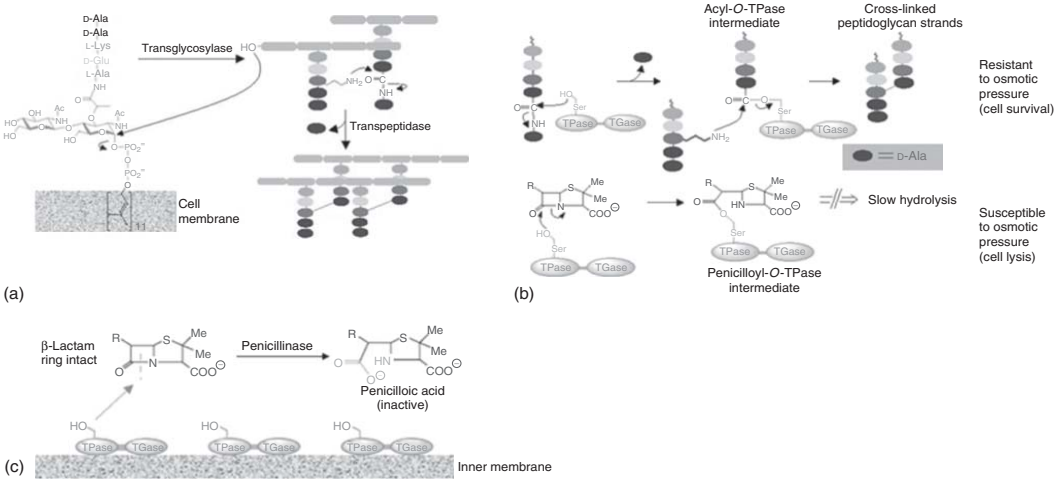

Figure 4.2 Transpeptidation inhibition during cell-wall biosynthesis by β-lactam antibiotic penicillins. (a) Cell-wall biosynthesis steps. (b) Inhibition of transpeptidase activity by penicillins through formation of a slowly hydrolyzing penicilloyl-O-TPase intermediate. This intermediate, deacylating very slowly, is unable to cross-linking promptly peptide chains in the peptidoglycan layer, leaving it mechanically weak and susceptible to lysis due to osmotic pressure changes. (c) Hydrolysis of β-lactam ring by β-lactamase penicillinase. TGase, transglycosylase; TPase, transpeptidase. *Source*: Modified from Ref. [11] with permission.

4.2.1.4 Proteases

Lantibiotics (i.e., lanthionine-containing antibiotics) are antimicrobial peptides, produced by a large number of gram-positive bacteria, which exert their antibiotic activity mainly by inhibiting bacterial cell-wall biosynthesis. The lantibiotic nisin, a 34-residue peptide produced by *Lactococcus lactis* strains, is widely used as a food preservative because of its potent bactericidal activity. In nisin-producer *L. lactis* strains, the lipoprotein NisI and the ABC tranporter system NisEFG prevent nisin toxic effect. In non-nisin-producing *L. lactis*, nisin resistance (*nsr*) could be conferred by *nsr* gene, which encodes a 35-kDa protein (NSR) able to digest nisin, thus reducing its affinity for its cellular target (the membrane-anchored cell-wall precursor lipid II) and, thus, its bactericidal activity [33].

4.2.2 Antibiotic Transferases Prevent Target Recognition

Transferases represent the largest family of resistance enzymes [7, 12]. These enzymes covalently modify antibiotics, impairing target binding. Their activities include *O*- and *N*-acylation, *O*-phosphorylation, *O*-nucleotidylation, *O*-ribosylation, *O*-glycosylation and thiol transfer. All these reactions require a cosubstrate, including adenosine triphosphate (ATP), acetyl-CoA, nicotinamide adenine dinucleotide (NADH) uridine diphosphate (UDP) glucose or glutathione, and, consequently, all these enzymes work only in the cytosol [7, 12].

4.2.2.1 Acyltransfer

Covalent modification by acyltransfer, in particular acetyltransfer, is a common mechanism of antibiotic inactivation employed by bacteria. Acetyltransferases target hydroxyl (for *O*-acetylation) and/or amine groups (for *N*-acetylation) on antibiotics and the resulting ester or amide is biologically stable and essentially irreversible without the action of a cognate esterase or amidase [7, 12].

Aminoglycoside Acetyltransferases The aminoglycoside antibiotics impair the codon–anticodon decoding mechanism by binding to 16S rRNA at the A-site of the ribosome. This interaction causes the inhibition of translation and also the synthesis of aberrant proteins as a consequence of translational infidelity (miscoding). The aminoglycoside acetyltransferases (AACs) modify the key hydroxyl and amine groups of the aminoglycoside antibiotics (Figure 4.3), blocking the interaction with the rRNA and resulting in resistance [12]. Aminoglycoside inactivation via AAC enzymes was the second bacterial-resistance mechanism discovered after that of penicillinases [34]. The AACs are classified according to their regiospecificity of acetyltransfer on the aminoglycoside structure [7, 12]. For example, the AAC(6′) acts by *N*-acetylating the aminoglycoside on the amine group frequently found at position 6′ of the aminohexose linked to position 4 of the central 2-deoxystreptamine ring, while the AAC(3) *N*-acetylates the amine group linked to position 3 of the 2-deoxystreptamine ring [7, 12]. Genes encoding these enzymes are widespread both in clinics (as a result of their frequent association with resistance

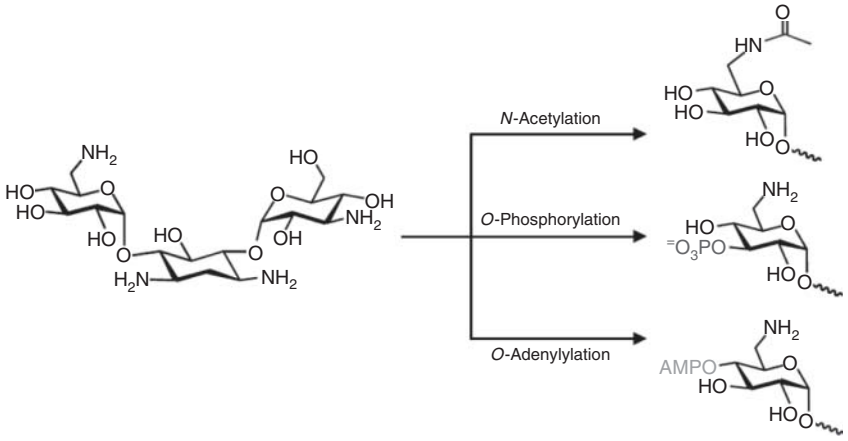

Figure 4.3 Aminoglycoside enzymatic modifications negatively affect ribosomal target recognition. Kanamycin can be chemically modified by three kinds of enzymatic processing: *N*-acetylation; *O*-phosphorylation; and *O*-adenylylation. *Source*: Modified from Ref. [11] with permission.

plasmids, transposons and integrons) and in the environment (as orthologs have been identified in many of bacterial genomes) [12].

Chloramphenicol Acetyltransferases Chloramphenicol prevents protein chain elongation by specifically binding to the 23S rRNA of the 50S ribosomal subunit, thereby inhibiting peptidyl transferase activity of the bacterial ribosome. Chloramphenicol acetyltransferases (CATs) are trimeric enzymes that have two distinct structural types: class A and class B (also known as the *xenobiotic CATs*) [7, 12, 35]. CATs inactivate chloramphenicol by covalently linking one or two acetyl groups, derived from acetyl-S-coenzyme A, to the hydroxyl groups. The chloramphenicol acetylation inhibits binding to the 23S rRNA.

4.2.2.2 Phosphotransferases

Aminoglycoside Phosphotransferases Kinases are enzymes catalyzing phosphate transfer from nucleotide triphosphates (NTPs), typically ATP, to a diverse set of substrates. Aminoglycoside phosphotransferases (APHs) (Figure 4.3), widely distributed among bacterial pathogens, are classified on the basis of their regiospecificity of phosphoryl transfer and substrate specificity [7, 12]. Thus, each APH is specific to a given range of aminoglycosides, which become unable to bind to their target on the A-site of the ribosome after phosphorylation. The genes encoding APH are frequently found on multidrug resistance R plasmids, transposons and integrons; therefore, the resistance genes are very often present in bacterial populations [7, 12]. The APH(3′) family is ubiquitous and is widely used as resistance markers in molecular biology research (e.g., the *neo* cassette).

Macrolide Phosphotransferases Phosphate transfer is also adopted by bacteria to block the effects of macrolides such as erythromycin. Characterization of the product of inactivation revealed that phosphorylation occurs on the free hydroxyl (site 2′ in the macrolide nomenclature) of the desosamine sugar that interacts directly with the 23S rRNA [7, 12]. Genes encoding macrolide phosphotransferase (MPH) enzymes have been isolated from *E. coli* (*mphA* and *mphB*) [36, 37] and from *S. aureus* (*mphC*) [38]. The presence of these genes results in very high MIC values ($2\,mg\,ml^{-1}$) for 14- and 16-member macrolides [12].

4.2.2.3 Nucleotidyltransferases

Nucleotidyltransferases, transferring nucleotide monophosphate moiety from NTPs to an accepting hydroxyl group on the antibiotic, are grouped in two major classes according to specificity of their target: (i) the ANTs that modify aminoglycosides (Figure 4.3) and (ii) the Lin proteins that inactivate the lincosaminide antibiotics that include lincomycin and its semisynthetic derivative, clindamycin [7, 12].

The aminoglycosides gentamicin and tobramycin, widely used in clinics, are both modified by ANT(2′) whose encoding gene is distributed among pathogenic bacteria [39].

Clindamycin is the lincosamide antibiotic most often used clinically. It binds to the peptide exit tunnel of the bacterial ribosome in the same region as the macrolide antibiotics [7]. There are three characterized lincosaminide nucleotidyltransferase genes, *linA* from *Staphylococcus haemolyticus*, *linA′* from *S. aureus* and *linB* from *Enterococcus faecium* [12, 40–42]. LinA and LinB do not show sequence homology and LinB modifies lincomycin and clindamycin at same position, while LinA modifies lincomycin and clindamycin at different positions [12].

4.2.2.4 ADP-Ribosyltransferases

Adenosine diphosphate (ADP)-ribosyl transfer, requiring NAD as ADP-ribosyl donor, is a common mechanism of protein posttranslational modification in both eukaryotes and prokaryotes. However, ADP-ribosylation of the RNA polymerase inhibitor rifampin (rifampicin), used in the treatment of infections caused by *Mycobacterium tuberculosis*, is so far the only well-documented example of this kind of modification in antibiotic resistance [12]. In mycobacteria, a unique rifampin ADP-ribosyltransferase (ARR) interferes with the activity of this drug [12, 43]. ARR-2, another enzyme with similar activity, is associated with multidrug resistance integrons in gram-negative bacteria [12, 44]. These enzymes, sharing about 55% identity to each other, are unique among ARRs for their small size and for their sequence differences with respect to other ARRs [7, 12].

4.2.2.5 Glycosyltransferases

Glycosyltransfer is a widespread mechanism of antibiotic resistance among soil bacteria, both producer and nonproducer strains, but infrequently encountered among pathogens [7, 12]. The soil bacterium *Streptomyces lividans* possesses the *mtg* gene, which is an example of this class of resistance [45]. The Mtg enzyme catalyzes glucosylation of erythromycin and other macrolides at position 2′ of

the desosamine sugar using UDP glucose as the glucose donor. Glycosylation of rifampin at position 23 by pathogenic *Nocardia* spp. is also reported but the enzyme has not yet been characterized [46].

4.2.3 Redox Enzymes

Oxidation is a common mechanism for mammalian detoxification of xenobiotics by a membrane-bound cytochrome P-450, which possesses broad substrate specificity. In contrast, the oxidation or reduction of antibiotics has not been frequently exploited by pathogenic bacteria [12]. The best-studied example of this strategy is the oxidation of tetracycline antibiotics by TetX, an enzyme that catalyzes the monohydroxylation of tetracycline antibiotics in an oxygen-dependent manner [47]. The gene encoding TetX was found on conjugative transposons in the obligate anaerobe *Bacteroides fragilis* and its role was only uncovered when the gene was cloned into *E. coli* [48, 49]. TetX acts on first- and second-generation tetracyclines and it is also active against the third-generation antibiotic tigecycline. Under aerobic conditions, TetX utilizes nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of magnesium and converts tigecycline to 11a-hydroxytigecycline. The modified molecule binds weakly magnesium, which is essential for its binding to ribosome [50].

Another predicted monooxygenase with antibiotic inactivation properties was cloned from rifampin-resistant *Rhodococcus equi*. Expression of the gene in *E. coli* resulted in rifampin resistance by an uncharacterized mechanism [12, 51].

4.3 Antibiotic Target Alteration: The Trick Exists and It Is in the Genetics

Alteration of the antibiotic target as a result of mutation, chemical modification, substitution, and masking of key binding elements, is a widespread strategy to elude antibiotic action.

4.3.1 Low-Affinity Homologous Genes

Spontaneous mutation is the driving force of molecular evolution. As a consequence of selective pressure in the modern antibiotic era, many cases of mutation not affecting bacterial fitness in housekeeping genes are reported to lead to resistance in previously susceptible strains. In addition, many strains are reported to be resistant to a class of antibiotics as a result of sequence differences in the target gene, which makes the product unable to interact with the antibiotic. This may be the case of antibiotic producer bacteria, such as actinomycetes, which have to protect themselves from the killing activity of their own product [52]. However, it is quite surprising that paralogous genes encoding products not susceptible to

antibiotics are found much more frequently in nonproducer bacterial strains [52]. In this case, the binomial chance-necessity concept (e.g., random mutations spread by means of selective pressure) could justify the hypothesis of an early exposure to toxic compounds in an early phase of bacterial molecular evolution. In this context, the isolation of environmental bacterial strains, not producing antibiotics and carrying paralogous genes whose products are not affected by the drugs, may be considered as a strong indication of the occurrence of a molecular struggle that started outside clinics [17].

4.3.1.1 Rifamycin Low-Affinity RpoB

Rifampicin inhibits DNA-dependent RNA polymerase in bacterial cells by binding its β-subunit, thereby compromising messenger RNA synthesis. In particular, rifampicin interacts with the β-subunit when the RNA polymerase is an $\alpha_2\beta$ trimer. Thus, rifampicin-resistant bacteria, including the producer strain *Amycolatopsis mediterranei*, posses RNA polymerases with different β subunit structures that are not readily inhibited by the drug [53]. In particular, most mutations map to the N-terminal region of resistant RpoB spanning amino acids 505–537 (*E. coli* numbering). The mutations are mainly point mutations resulting in single amino acid substitutions, with few deletions or insertions, causing poor binding of rifampicin to the RNA polymerase [53].

4.3.1.2 Mutated Genes Conferring Resistance to Quinolone, Fluoroquinolone and Aminocoumarins

Quinolone and fluoroquinolone interfere with DNA replication. A high level of resistance to this class of antibiotics is associated with mutations in the *gyrA gene*, encoding a subunit of DNA gyrase, in gram-negative bacteria and in *gyrA* and *parC* (a subunit of topoisomerase IV) in gram-positive bacteria [54, 55]. A 41 amino acid sequence, corresponding to amino acids 67–106 in *E. coli* GyrA, was identified in both gram-negative and gram-positive organisms as the quinolone-resistance-determining region [55].

Aminocoumarins, such as novobiocin, are inhibitors of bacterial DNA gyrase. In particular, aminocoumarins target the GyrB subunit, necessary for energy transduction. Resistance to this class of antibiotics usually results from genetic mutation in the *gyrB* subunit [56].

4.3.1.3 PBP2a: A Low-Affinity Penicillin-Binding Protein

The *mecA* gene encodes the penicillin-binding protein 2a (PBP2a) a transpeptidase membrane protein that possesses a low affinity for β-lactam antibiotics, such as methicillin and penicillin, and is responsible for β-lactam resistance in methicillin-resistant *S. aureus* (MRSA) [54]. The *mecA* gene expression is controlled by *mecI*, encoding a negative regulator, and *mecR1*, encoding a sensor protein, which derepress *mecA* expression inactivating MecI in the presence of β-lactam. The *mecA* gene is placed in the staphylococcal chromosomal cassette *mec* (SCC*mec*), which is a mobile genetic element of the *Staphylococcus* bacterial species that contains the *ccr* genes coding for recombinases required for horizontal transfer [2, 20, 54].

4.3.1.4 Dihydropteroate Synthases Not Inhibited by Sulfonamide

Sulfonamides, synthetic antimicrobial agents that contain the sulfonamide group, act as competitive inhibitors of dihydropteroate synthetase (DHPS), an enzyme involved in folate synthesis. Sulfonamide resistance in gram-negative bacilli generally arises from the acquisition of genes encoding dihydropteroate synthase variants such as *sul1, sul2* and *sul3* that are not inhibited by the drug [57]. The *sul1* and *sul3* genes are normally found linked to other resistance genes in class 1 integrons, while *sul2* is usually located in small nonconjugative plasmids or in large transmissible multiresistance plasmids [57].

4.3.2 Chemical Modification of Antibiotic Target

The capability to chemically modify a molecular target of an antibiotic is mainly attributed to gene products expressed in antibiotic-producing bacteria such as actinomycetes. Nevertheless, homologous genes were recently found in resistant strains from clinical isolates. The spreading of such genes represents a problem from a nosocomial viewpoint and poses intriguing questions concerning the evolutionary history of resistance genes.

4.3.2.1 23S rRNA Modification

Erythromycin, a natural product of *Saccharopolyspora erythraea*, was the first macrolide to be advanced to medical use in the early 1950s for the treatment of infections due to gram-positive pathogenic bacteria [58]. Macrolides inhibit bacterial growth by binding to the ribosome and blocking the nascent polypeptide chain in the early rounds of protein synthesis [59] or in some cases macrolides with extended side chains reach close to the catalytic center and stop peptide bond formation from the beginning [59]. Erythromycin methyltransferases (Erms) from macrolide-resistant bacteria, including ErmE from erythromycin producer *S. erythraea*, can methylate adenine at position 2058 of 23S rRNA (*E. coli* numbering) [60]. The *ermA* gene, carried by Tn554-like transposons, is widespread in MDR-MRSA strains while *ermC*, usually plasmid-located, is more common among methicillin-sensitive *Staphlococcus aureus* (MSSA) strains [20]. In *Staphylococci*, *erm* genes can also confer resistance to a broader group of antibiotics such as lincosamide and streptogramin in addition to macrolides (macrolide-lincosamide-streptogramin B, MLSB resistance) [20].

4.3.2.2 16S rRNA Modification

Resistance to aminoglycosides is frequently due to the acquisition of modifying enzymes such as acetyltransferases, phosphorylases and adenylyltransferases (Section 4.2). Other mechanisms of aminoglycoside resistance include single-step mutations in chromosomal genes encoding ribosomal proteins, impaired antibiotic uptake and ribosomal protection by methylation of 16S rRNA [61]. Methylation of bases involved in the binding of aminoglycosides to 16S rRNA leads to a reduction in binding affinity, thereby causing high-level resistance to

aminoglycosides. Methylases, such as KamB and KamC, are intrinsically produced by some aminoglycoside-producing organisms such as *Streptomyces* spp. and *Micromonospora* spp. [62]. Recently, several plasmid-encoded 16S rRNA methylases have emerged in clinical isolates of gram-negative bacilli [61]. The ArmA, RmtA and RmtB methylases were detected in *P. aeruginosa* strains and a *Serratia marcescens* strain, respectively [63].

4.3.2.3 Reprogramming Chemical Composition of a Bacterial Cell-Wall Precursor

Glycopeptides, nonribosomally synthesized peptides, target the D-Ala-D-Ala end of uncross-linked pentapeptide side chain in nascent peptidoglycan chains. The interaction, preventing the transpeptidase recognition, inhibits peptide cross-linking, causing the formation of a weak cell wall that is not able to withstand the osmotic pressure (Figure 4.4) [10, 11]. A sophisticated example of the strategy to escape the glycopeptide effect was revealed in both glycopeptide-producing and in nonproducing bacteria, such as vancomycin-resistant enterococci (VRE) [10, 11]. In these strains, the *vanHAX* operon genes encode a set of enzymes that reduces pyruvate to D-lactate (VanH), adds D-alanine and D-lactate together to produce D-Ala-D-Lac (VanA) and then hydrolyses the D-Ala-D-Ala (VanX) (Figure 4.4 and Figure 4.5) [10, 11]. The resistance mechanism is positively regulated by a two-component signal transduction system (*vanS* and *vanR* genes) in the presence of vancomycin (Figure 4.5) [10, 11]. The D-Ala-D-Lac is incorporated into the end of the peptidoglycan strands instead of D-Ala-D-Ala and this substitution, having no effect on the cross-linking efficiency, lowers the binding affinity of vancomycin by 1000-fold and enables the VRE to grow at 1000-fold higher levels of antibiotic (Figure 4.4) [10, 11]. The high homology between glycopeptide-resistance determinants suggests horizontal transfer events from producer to nonproducer strains.

4.3.3 Ribosomal Protection and Tetracycline Resistance

Resistance to tetracycline may be mediated by inactivation by TetX (Section 4.2.3) or by the integral membrane efflux protein tetracycline (TetA) (Section 4.4.2) or by a mechanism known as *ribosomal protection* mediated by a soluble protein [64]. Ribosomal protection proteins (RPPs) are 72.5 kDa proteins belonging to a widely distributed class of tetracycline resistance determinants. There are 11 different types of RPPs in both gram-positive and gram-negative bacteria [64]. TetO and TetM are the most prevalent and the best-studied classes of RPPs, while OtrA is believed to be the ancestor of some other RPPs found in pathogens such as *Mycobacteria*. RPPs display high homology to translation elongation factors EF-Tu and EF-G, which are ribosome-dependent GTPases. Therefore, it has been suggested that RPPs are EF paralogs that have evolved through duplication and divergence of an ancestral GTPase [64]. RPPs were earlier proposed to work as tetracycline-resistant elongation factors capable of carrying out protein synthesis in the presence of tetracycline, but now it is believed that RPPs displace tetracycline from the ribosome so that

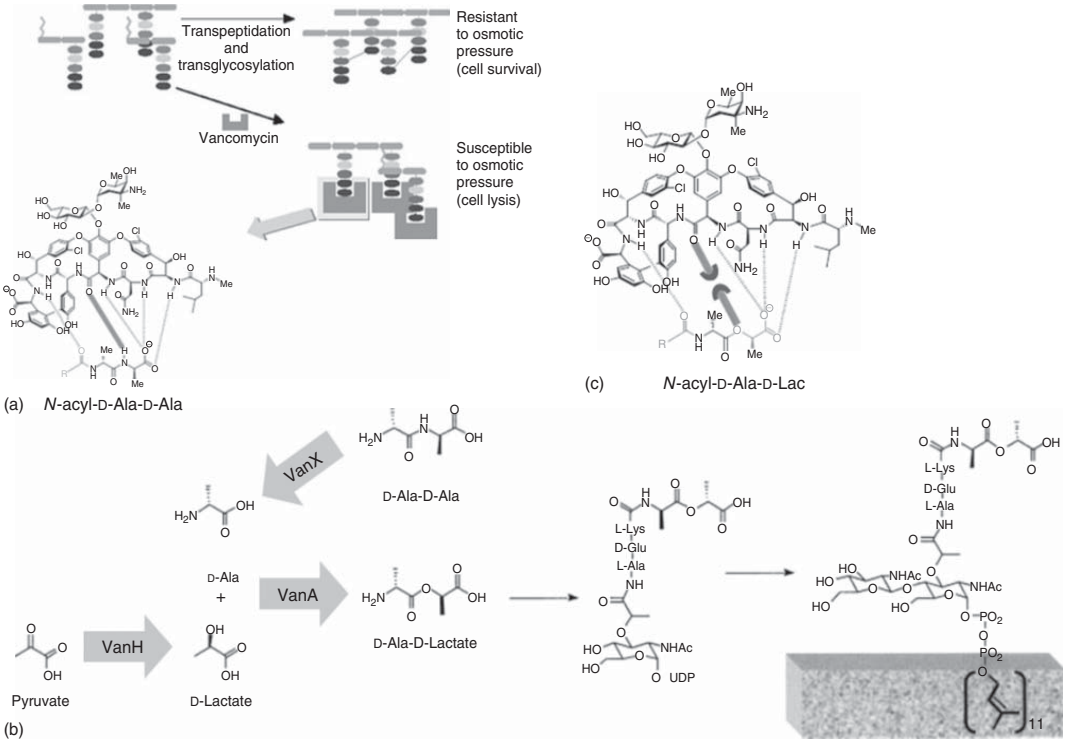

Figure 4.4 Transpeptidation inhibition during cell-wall biosynthesis by vancomycin. (a) Inhibitory effect of vancomycin on cross-linking and strength-conferring transpeptidation by sequestering of substrate. (b) Reprogramming of biochemical composition of bacterial cell-wall precursors by *vanHAX* gene products. (c) Complexation of the D-Ala-D-Ala termini of peptidoglycans by vancomycin in a network of five hydrogen bonds. *Source*: Modified from Refs. [10, 11] with permission.

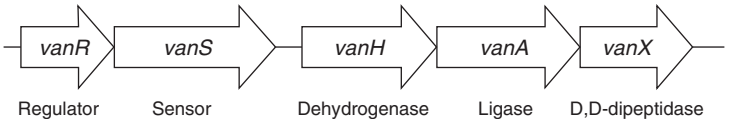

Figure 4.5 Schematic representation of *van* genes in vancomycin-resistant enterococci.

the tetracycline-free ribosome can bind the aminoacyl-tRNA (aa-tRNA) in the A-site and protein synthesis can continue [64]. RPPs are effective against first- and second-generation tetracyclines but not against tigecycline, which is a third-generation compound probably because this drug has a stronger binding affinity for its target [64].

4.3.4
Chromosomal Mutations in Genes Required for Membrane Phospholipid Metabolism: Lipopeptide Resistance

The lipopeptide antibiotic daptomycin, produced by actinomycete, is used to treat gram-positive bacterial infections, including those caused by enterococci and staphylococci [65]. Daptomycin is approved to treat complicated skin and skin structure infections and has been used to treat VRE bacteremia and endocarditis, among other infections [20, 66]. It is proposed that daptomycin kills cells by a calcium-dependent insertion into the cell membrane followed by oligomerization that causes pores, allowing ion leakage from the cell and rapid depolarization of the bacterial cell membrane [67]. Daptomycin resistance has been extensively studied in *S. aureus*, where it results from chromosomal mutation. Microarray-based comparative genome analyses of *S. aureus* and *E. faecalis* strains subjected to *in vitro* daptomycin serial passage revealed that certain genes and intergenic regions (such as *mprF*, *rpoB*, *yycG* and *cls*) acquired mutations during the evolution of daptomycin resistance. Mutations in these regions in many, but not all, daptomycin-resistant *S. aureus*, *E. faecalis* and *E. faecium* clinical isolates have also been detected. The impact of these genetic changes has not been fully delineated [66, 68]. MprF catalyzes the lysinylation of phosphatidylglycerol (PG), generating lysylphosphatidylglycerol (Lys-PG). As daptomycin seems to interact preferentially with PG, the binding of lysine, which would convert negatively charged PG to positively charged Lys-PG, may interfere with daptomycin-membrane interactions [64]. Cls catalyzes reversible transphosphatidylation of cardiolipin (CL; bis-PG), a negatively charged phospholipid associated with septal and polar membrane protein-lipid microdomains in *B. subtilis* and other bacteria. CL has the potential to significantly impact local membrane structure and charge–charge interactions at the membrane. Thus, *cls* mutations observed in the daptomycin-resistant strains theoretically could result in decreased CL synthesis or increased CL degradation, thus changing CL amount in membranes of daptomycin-resistant enterococci [66]. Therefore, membrane composition seems to be critical for daptomycin antibiotic activity and, therefore, an improved understanding of how membrane compositions

change in resistant strains would be critical for unraveling the precise mechanism of daptomycin resistance.

4.3.5 Covalent Modifications on Lipopolysaccharide Core Conferring Polymixine Resistance

The PmrA–PmrB two-component system governs resistance to antimicrobial peptide compounds including polymyxin, polylysine, protamine and neutrophil antimicrobial peptides CAP37 and CAP57 [69]. The genes encoding these peptides have been shown to be activated *in vivo* and are regulated by PhoP–PhoQ system, but can also be activated under mild acidic conditions in a PhoP–PhoQ independent manner. PmrA–PmrB activation results in the modification of phosphate groups of the lipopolysaccharide (LPS) core and lipid A with ethanolamine and modification of the 4′ phosphate of lipid A with aminoarabinose. These covalent modifications, seen in resistant bacteria including *Yersinia enterocolitica*, *Proteus vulgaris*, *E. coli*, *K. pneumoniae* and *Burkholderia* (*Pseudomonas*) *cepacia*, reduce electrostatic interactions and, hence, weaken binding between the peptide and the cell surface [69]. PmrA–PmrB are involved in the regulation of the *pmrA-pmrB* operon itself and of *pmrE* and *pmrF* loci that are necessary for resistance to polymyxin [69]. The *pmrE* locus contains a single gene previously identified as *pagA* (or *ugd*), which encodes a UDP-glucose dehydrogenase [69]. The *pmrF* locus comprises the second gene of a putative operon predicted to encode seven proteins, some with similarity to glycosyltransferases and other complex carbohydrate biosynthetic enzymes involved in lipid A aminoarabinose modification. The activity of these enzymes can promote resistance to cationic antimicrobial peptides [69]. In addition, genes flanking this putative operon are also regulated by PmrA–PmrB and/or have been associated with *S. typhimurium* polymyxin resistance with a mechanism that is still to be investigated [69].

4.4 Efflux Systems

Efflux pumps are major players in bacterial MDR and pose major hurdles in the drug discovery process [70–76]. They typically export structurally different organic compounds including antibiotics, environmental toxic compounds, or molecules produced by the host organism such as bile, indicating that these systems could allow bacteria to survive in their ecological niche.

Efflux pumps can be specific to one substrate or can transport a range of unrelated substances; the efflux pumps that transport multiple, structurally dissimilar toxic agents can be associated with MDR. Therefore, MDR efflux pumps are of clinical relevance because they can render a bacterial infection untreatable by the antibiotics of choice. MDR efflux pumps are found in all bacteria and their primary functions could be other than antibiotic resistance such as maintenance of cell homeostasis

or intracellular solute concentrations, extrusion of toxic by-products of metabolism and transport of nucleotides or amino acids.

Efflux pumps reduce the intracellular antibiotic concentration and often act synergistically with other resistance mechanisms to provide a high level of resistance to antibiotics. Efflux-pump genes are mostly located on the chromosome, although they can also be carried by plasmids, and are frequently subjected to both specific and global regulation.

Bacterial efflux pumps are grouped into five families (Figure 4.6) according to their primary structure and mode of energy coupling:

1) ATP-binding cassette (ABC) superfamily;
2) major facilitator superfamily (MSF);
3) small multidrug-resistance family (SMR);
4) resistance-nodulation-division (RND) superfamily;
5) multidrug and toxic compound extrusion (MATE) family.

These families, except for the ABC family, are secondary transport systems and utilize an electrochemical gradient of cations across the membrane for drug transport.

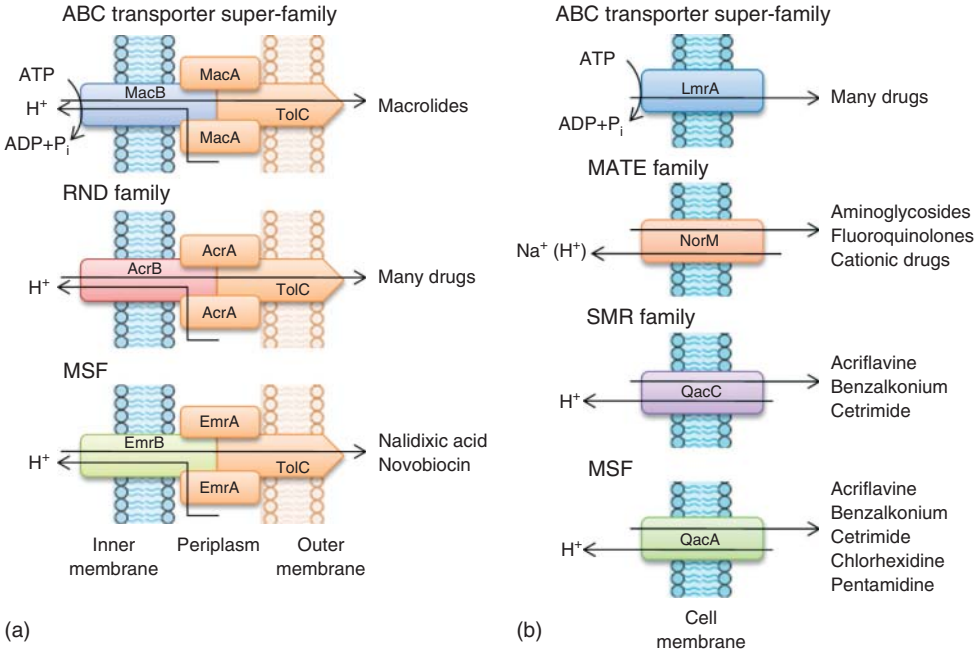

Figure 4.6 Diagrammatic representation of the structure and membrane location of members of the five characterized families of multidrug resistance efflux pumps in gram-negative (a) and gram-positive (b) bacteria. IM, inner membrane; P, periplasm; OM, outer membrane; CM, cell membrane.

Efflux pumps usually consist of a monocomponent protein with transmembrane spanning domains; however, in gram-negative bacteria an efflux pump, located in the inner membrane, works together with a periplasmic protein named membrane-fusion protein (MFP) and an outer membrane channel protein.

A bacterial cell can express efflux pumps from more than one family and/or more than one type of pump belonging to the same family.

4.4.1 The ATP-Binding Cassette (ABC) Superfamily

The transporters of the ABC family are conserved from humans to bacteria and export a wide array of substrates in a process driven by ATP hydrolysis [77, 78]. ABC transporters consist of a transmembrane domain (TMD) and a nucleotide-binding domain (NBD).

LmrA from *L. lactis* is the first member of the ABC transporter family discovered in bacteria, whose TMD and NBD are expressed as a single polypeptide. LmrA catalyzes the extrusion of many hydrophobic compounds including antibiotics through the cell membrane. ATP binds to the NBD, where binding and hydrolysis induce conformational changes that lead to the extrusion of the substrate via the TMD [79, 80].

S. aureus Sav1866 exporter protein is a homolog of LmrA that contains two nucleotide-binding domains in close contact and two TMDs; by simultaneous hydrolysis of two molecules of ATP, this protein opens a transmembrane channel and pumps drugs out of the cell, thereby conferring MDR [81–83].

E. coli MacB (Figure 4.6) is an ABC-type macrolide efflux transporter with four transmembrane segments and one nucleotide-binding domain, which functions by cooperating with the MFP MacA and the multifunctional major outer membrane channel TolC [84–86]. TolC plays an important role in the excretion of a wide range of molecules, including antibiotics, bile salts, organic solvents, enterobactin, several antibacterial peptides, and virulence factors [87].

4.4.2 The Major Facilitator Superfamily (MSF)

The MSF is a very large, ancient group of proteins consisting of secondary integral membrane transporters driven by chemiosmotic energy [88] and includes proton (H^+)/drug antiporters such as QacA, NorA, NorB, NorC and LmrS of *S. aureus*, Mdt(A) of *L. lactis*, MdfA and EmrA of *E. coli* and TetA family of efflux pumps from gram-negative and gram-positive bacteria. These proteins span the lipid bilayer of the cell membranes 12–14 times.

The QacA efflux pump [89–92] spans the membrane 14 times and is energized by H^+ to extrude structurally diverse monovalent and divalent cationic substrates, in particular quaternary ammonium compounds. In MDR *S. aureus*, QacA is encoded by plasmid-borne genes and the expression of *qacA* genes is regulated by QacR, a transcription regulator belonging to the *tetR* family [93].

S. aureus NorA efflux pump extrudes the quinolone drug norfloxacin and several antimicrobial agents including chloramphenicol [94, 95]. NorA, possessing 12 TM and being chromosomally encoded, is partly homologous to tetracycline resistance and sugar transport proteins [96, 97]. The *norA* cloned from chromosomal DNA of quinolone-resistant *S. aureus* TK2566 conferred relatively high resistance to hydrophilic quinolones such as norfloxacin, enoxacin, ofloxacin and ciprofloxacin, but only low or no resistance to hydrophobic drugs such as nalidixic acid, oxolinic acid and sparfloxacin in *S. aureus* and *E. coli*. NorB [98] and NorC [99], organized into 14 transmembrane segment (TMS), confer resistance to quinolones, such as norfloxacin, ciprofloxacin and sparfloxacin. The expression of *norA*, *norB* and *norC* together with *tet38*, which encodes tetracycline resistance, is under the control of MgrA, a global regulator that also affects diverse virulence factors [100, 101].

LmrS was identified in a clinically isolated MRSA strain [102]. Proteins homologous to LmsrS are widely distributed among gram-positive bacterial genera such as *Staphylococcus*, *Bacillus*, *Lactobacillus*, *Listeria* and *Enterococcus*. LmrS confers resistance to linezolid and fusidic acid, two antimicrobials with strong activity against MRSA. This efflux pump, with 14 TM, is encoded by a chromosomal *lmrS* gene. The cloned *lmrS* gene confers resistance to kanamycin, lincomycin, fusidic acid, linezolid, chloramphenicol, erythromycin, streptomycin, trimethoprin and florfenicol.

The plasmid-specified multiple drug transporter Mdt(A) contains 12 TMS and is a member of the MSF with some interesting structural differences; it has two antiporter motifs and a putative ATP-binding site [103]. Mtd(A) confers resistance to lincosamides, 14-, 15- and 16-membered macrolides, streptogramins and tetracyclines. The molecular mechanism responsible for drug transport by Mdt(A) remains to be elucidated.

MdfA [104, 105], identified in *E. coli*, contains 12 TMS. *E. coli* cells expressing MdfA from a multicopy plasmid exhibit resistance not only to lipophilic compounds including ethidium bromide, daunomycin, tetraphenyphosponium, rhodamine, rifampin, tetracycline, puromycin but also to chemically unrelated clinically important antibiotics such as erythromycin, choramphenicol, some aminoglycosides and fluoroquinolones. In addition, MdfA is involved in maintaining the physiological pH of the cell.

EmrB is a membrane protein with 14 TM domains, while the MFP EmrA has a large soluble C-terminal domain with a single N-terminal TM domain; together with the outer membrane channel TolC, EmrAB forms a tripartite efflux system [106].

Efflux of tetracyclines predominantly occurs via proteins that are members of the major facilitator superfamily (MFS) group of integral membrane transporters. There are 26 different classes of MFS tetracycline transporters present in gram-negative and gram-positive bacteria.

The *tetA* family pumps are grouped into two major groups [107, 108]. The first group comprises chromosomally encoded efflux pumps possessing 12-TMS, found in gram-negative bacteria. The second group comprises plasmid-encoded

efflux pumps having 14-TMS identified in *S. aureus*, *Bacillus*, *Staphylococcus* and *Streptococcus* spp. [73, 109].

In *E. coli*, tetracycline enters cells by simple diffusion through the lipid bilayer region of the plasma membrane as a protonated neutral form. Then it loses a proton and chelates with Mg^{2+}. The resulting monovalent cation is exported by TetA coupled with H^+ influx. Thus, this pump functions as a metal-tetracycline/H^+ antiporter [110].

4.4.3 The Small Multidrug-Resistance Family (SMR)

This family of transporters is represented by EmrE of *E. coli* and QacC of *Staphylococcus epidermidis* [111–114]. The SMR are small (about 12 kDa) integral inner membrane proteins conferring resistance to lipophilic compounds, like quaternary ammonium compounds, and to a wide range of antibiotics, such as β-lactams, cephalosporins and aminoglycosides [74].

These proteins span the cytoplasmic membrane as four transmembrane α-helices with short hydrophilic loops, making them hydrophobic and permitting their solubilization in organic solvents. Similar to the MSF superfamily proteins, the SMR proteins perform drug efflux via an electrochemical H^+ gradient. The SMR family contains more than 250 annotated members and is grouped into three subclasses: (i) the small multidrug pumps (SMPs), (ii) the paired small multidrug-resistance proteins (PSMR) and (iii) the suppressors of GroEL mutant proteins (SUG). The latter do not carry out drug efflux but their overaccumulation suppresses GroEL mutations, suggesting that SUG proteins may play an important role in the uptake of chaperone regulatory compounds. The peptide methionine sulfoxide reductase (PMSR) proteins are distinct from SMP and SUG subclass proteins because they are constituted by two SMR homologs that must be simultaneously expressed to confer drug resistance. PMSR protein pairs generally consist of one protein with typical SMR protein length and of a second longer protein, for example, *E. coli* YdgE and YdgF or *B. subtilis* EbrA and EbrB [115–117]. PSMR proteins are structurally different from other SMR homologs owing to the presence of longer hydrophilic loops and of a large hydrophilic C-terminus in one of the two proteins. SMR proteins may be encoded on the chromosomes or on plasmids and may be associated with integrons.

4.4.4 The Resistance-Nodulation-Division (RND) Superfamily

Efflux pumps of the RND family, which function as H^+/drug antiporters, are mainly found in gram-negative bacteria and catalyze the active efflux of many antibiotics and chemotherapeutic agents [118]. RND transporters are protein complexes that span both cytoplasmic and outer membrane. The complex comprises a cytoplasmic membrane transporter protein, a periplasmic-exposed membrane adaptor protein classified as MFP, and an outer membrane channel protein. Importantly, each of

these three component proteins is essential for drug efflux and the absence of even one component makes the complex nonfunctional.

The *E. coli* AcrAB-TolC and the *P. aeruginosa* MexAB–OprM complexes are well characterized [119–121]. AcrAB-TolC can handle a very wide range of compounds. These include cationic dyes, detergents, bile acids and antibiotics such as penicillins, cephalosporins, fluoroquinolones, macrolides, chloramphenicol, tetracyclines, novobiocin, fusidic acid, oxazolidinones and rifampicin. The MexAB-OprM complex exports antimicrobial compounds, such as fluoroquinolones, β-lactams, tetracycline, macrolides, choramphenicol, novobiocin, trimetropin and sulphonamides, and also exports dyes, detergents, disinfectants, organic solvents and acylated homoserine lactones involved in quorum sensing.

The AcrB or MexB transporter protein captures its substrates, either from within the phospholipid bilayer of the inner membrane or from the cytoplasm, and then transports them to the extracellular medium through TolC or OprM, respectively, which form a channel in the outer membrane. Cooperation between the inner membrane transporter proteins and outer membrane channel proteins is mediated by periplasmic accessory proteins AcrA and MexA, respectively. Thus, in enterobacteriaceae TolC can function as a channel for different RND-family efflux pumps and can interact with ABC and MFS transporters. Similarly, OprM of *P. aeruginosa* can interact with various RND-family proteins.

4.4.5
The Multidrug and Toxic Compound Extrusion (MATE) Family

The MATE family is the most recently categorized among the five efflux transporter families [122, 123]. The MATE family has been shown to be ubiquitously distributed and extremely flexible in function. MATE efflux pumps utilize Na^+/H^+ gradient for transport of metabolic and xenobiotic organic cations and have been reported to contain three branches: the NorM branch, a branch containing several eukaryotic proteins and a branch containing *E. coli* DinF.

These proteins are predicted to have 12 α-helical transmembrane regions. The X-ray structure of the NorM revealed an outward-facing conformation with two portals open to the outer leaflet of the membrane and a unique topology of the predicted 12 transmembrane helices distinct from any other known MDR transporter.

NorM, a multidrug Na^+-antiporter, was the first MATE family pump identified from *Vibrio parahaemolyticus*. It confers resistance to dyes, fluoroquinolones and aminoglycosides. NorM homologs have recently been characterized in many species such as *E. coli, N. gonorrhoeae, V. cholerae* and *Erwinia amilovora* [124–127].

The DinF protein is an uncharacterized member of this family of transporters. Expression of the *dinF* gene is DNA damage (UV or mitomycin C) inducible. The *dinF* gene is located downstream of the *lexA* gene, which encodes the global repressor of the SOS regulon. On the basis of sequence similarity, DinF may function as a proton-driven efflux system, possibly for nucleotides, given its potential role in response to DNA damage [128, 129].

As the majority of the bacterial MATE transporters have been identified by expression in *E. coli*, the functional role of these pumps in the native hosts is unclear.

4.5 The Case Stories of Intrinsic and Acquired Resistances

4.5.1 β-Lactam Resistome of *P. aeruginosa*: Intrinsic Resistance Is Genetically Determined

P. aeruginosa is an opportunistic pathogen showing low intrinsic antibiotic susceptibility. Intrinsic resistance is attributed to the low permeability of cellular envelopes together with the presence of chromosomally encoded multidrug efflux pumps or antibiotic-inactivating enzymes that resemble those present in transposable elements and usually acquired by horizontal transferring. However, further intrinsic mechanisms act in synergy as many chromosomal genes that contribute to β-lactam resistance of *P. aeruginosa* were identified using a comprehensive library of transposon-tagged insertion mutants [18]. In particular, genes whose inactivation resulted in changes in antibiotic resistance encode proteins that belong to a variety of functional groups, including cell division (FtsK), metabolic enzymes such as phosphoenolpyruvate carboxikinase, elements involved in cell attachment and motility such as fimbrial proteins or chemotaxis proteins, elements involved in the biosynthesis of LPS and in alginate production, and transcriptional regulators like GlnK (involved in nitrogen metabolism) [18]. Other resistance elements such as transporters, porins and regulatory proteins involved in the expression of chromosomally encoded β-lactamases (similar to those encoded by *dacB, mpl, ampR*, and *ampD*) were also identified. Altogether, these results indicate that the intrinsic resistome of *P. aeruginosa* might be considered a property highly dependent on the metabolic networks and biochemical characteristics of cells and not just the consequence of bacterial adaptation to the presence of antibiotics [18].

4.5.2 Acquired Antibiotic Resistance in *S. aureus*

The rapid acquisition of resistance determinants in *S. aureus*, starting with penicillin and methicillin, up to the most recent linezolid, is an example of bacterial adaptive evolution of bacteria in the antibiotic era. Resistance mechanisms in *S. aureus* include enzymatic inactivation of the antibiotic (penicillinase and aminoglycoside-modification enzymes), modification of antibiotic target (PBP2a of MRSA and D-Ala-D-Lac of peptidoglycan precursors of vancomycin-resistant strains), trapping of the antibiotic (vancomycin) and efflux pumps (fluoroquinolones and tetracycline) [20].

4.5.2.1 Acquired Resistance to β-Lactams and Glycopeptides

When penicillin first entered into clinical use in the 1940s, all *S. aureus* isolates were virtually susceptible to this antibiotic. However, within 10 years, *S. aureus* strains resistant to penicillin appeared and soon spread to become the most frequently isolated strains. Since then, several new antibiotic classes have been used, but *S. aureus* has shown a unique ability to quickly respond to each new challenge with the development of a new resistance mechanism. *S. aureus* resistance is mostly acquired via horizontal DNA transfer. Penicillin resistance is due to the production of β-lactamase, whose encoding gene is carried by a plasmid. Plasmids encoding penicillinase production also carry other resistance genes, such as resistance to disinfectants (quaternary ammonium compounds), dyes (acriflavine and ethidium bromide) and heavy metals (lead, mercury and cadmium), as well as to other antibiotics (erythromycin, fusidic acid and aminoglycosides) [20]. After the emergence of β-lactam-resistant strains, methicillin was designed to be invulnerable to the hydrolytic activity of the staphylococcal enzyme [20]. However, some strains of *S. aureus* developed resistance to this antibiotic very soon after its use. Unlike MSSA strains, MRSA strains are often MDR ones, being resistant also to a number of antibiotics of different classes, including macrolides, aminoglycosides and fluoroquinolones [20]. As described in Section 4.3.1.3, methicillin resistance is due to the production of an additional penicillin-binding protein named PBP2a, which possesses a reduced affinity for penicillin and β-lactams. As described in Section 4.3.1.3, PBP2a is the product of the *mecA* gene, which is controlled by regulatory genes *mecI* and *mecR1*. The *mecA* complex, whose origin is unknown, is found within a 30–60 kb mobile genetic element, denominated SCCmec in which is also found a *ccr* gene complex containing two recombinase genes (*ccrA* and *ccrB*), which mediate site-specific integration/excision of the element from the staphylococcal chromosome. SCCmec is an antibiotic resistance island as it can integrate additional mobile elements or resistance genes including insertion sequences, transposons, such as Tn554, which carries resistance genes for spectinomycin and erythromycin, integrated plasmids, such as pUB110, which encodes tobramycin and kanamycin resistance, mercury operons and more [20]. After the widespread emergency of MRSA, vancomycin has represented the cornerstone of therapy for MRSA infections. Over the past decade, a long-feared event has occurred: the appearance of strains that are not susceptible to vancomycin, showing either intermediate resistance (vancomycin-intermediate *S. aureus* (VISA)) or, worse, full resistance to this antibiotic (vancomycin-resistant *S. aureus* (VRSA)) [20]. The intermediate resistance in VISA has been associated with the presence of a thickened cell wall rich in peptidoglycan chains that are not cross-linked. Thus, vancomycin bound to the terminal dipeptide D-Ala-D-Ala is unable to reach the inner cell-wall layers, where vancomycin can exert its inhibitory action, blocking the incorporation of the precursors into the nascent peptidoglycan [20]. No characteristic genetic trait has been associated with VISA, although a relationship was observed with the loss of the accessory gene regulator (*agr*) locus, a quorum-sensing gene cluster that regulates virulence, conferring a selective survival advantage in the presence of vancomycin [20]. Unlike VISA, VRSA are usually high-level vancomycin resistant. VRSA strains

have acquired the *vanHAX* operon (Figure 4.5) that confers high-level resistance to both glycopeptides, vancomycin and teicoplanin, from VRE [20]. Therefore, the genetic and biochemical bases of resistance are the same as those of VRE and have been completely elucidated [10, 11, 20].

4.5.2.2 Acquired Resistance to Fluoroquinolones

In *S. aureus*, resistance to fluoroquinolones is conferred by point mutations occurring primarily in the subunit ParC (also named GrlA) of topoisomerase IV and secondarily in the subunit GyrB of DNA gyrase [20]. In addition, in some strains, overexpression of an efflux pump termed *NorA* contributes to the resistance phenotype. Multiple mutations and combination of resistance mechanisms also confer cross-resistance to newer fluoroquinolones, including those with increased activity against gram-positive bacteria [20]. New antibiotics such as linezolid, which is very active against MRSA strains, have been recently used to treat MRSA infections. Linezolid is an antibiotic belonging to the new class of the oxazolidinones that inhibits protein synthesis by binding to domain V of the 23S subunit of the bacterial ribosome [20]. As the chemical structure and/or the mechanism of action of this new drug are novel, the occurrence of natural resistance or cross-resistance was not anticipated. However reports of resistance developing during linezolid treatment are increasing [20].

4.6 Strategies to Overcome Resistance

The molecular struggle between antibiotic resistance and susceptibility is an evolutionary force that speeded up in the clinical experience of the past 50 years. Anyway, the understanding of the dynamics driving the molecular evolution of antibiotic-resistance genes can be used to survey clinically relevant organisms for the emergence of resistance during therapy and/or to improve the strategies leading to drug discovery and optimization. In this context, databases unifying resistance gene information, such as the Antibiotic Resistance Genes Database (ARDB, *http://ardb.cbcb.umd.edu/index.html*), would be helpful for facilitating studies of antibiotic resistance genes and for developing strategies to overcome the antibiotic resistance emergency. In fact, the extensive knowledge of resistance mechanism can be exploited to chemically modify promising molecules in such a way as to avoid enzymatic modification in vulnerable hot spots or to codevelop enzyme-specific inhibitors of resistance.

For example, chemical modifications of hydroxyl groups that can be targeted by kinases which inactivate aminoglycoside antibiotics led to the development of aminoglycosides such as tobramycin and gentamicin that lacked sites of inactivation [12, 130]. A similar strategy was adopted for florfenicol to overcome CAT-mediated resistance by acetylation at the hydroxyl linked to C3 [35]. Chemical modification of molecular structure has also driven the development of new β-lactamase-insensitive semisynthetic β-lactams, such as penems and

carbapenems [12, 131]. These antibiotics possess broad-spectrum activity and enhanced stability to β-lactamases.

Another application of a thorough understanding of resistance mechanisms is the development of resistance enzyme inhibitors. These inhibitors can be coadministered with the antibiotics to maintain antimicrobial activity. This approach has been highly successful in clinics as exemplified by the use of the β-lactamase inactivators of clavulanic acid, sulbactam and tazobactam to overcome resistance to the β-lactamase penicillinases [12, 131]. Interestingly, inhibitors blocking eukaryotic Ser- , Thr-, Tyr-kinases were also able to interfere with aminoglycoside kinases [7, 133]. In addition, many regulatory proteins activating resistance genes are two-component systems where His-kinase is the sensor membrane protein activating the transcriptional regulatory proteins as in the case of VanS-VanR. The use of His-kinase inhibitors, which lack targets in eukarya cells, may result in decreased resistance toward glycopeptides with the absence of unwanted collateral effects. Many pharmaceutical companies possess chemical libraries of protein kinase inhibitors that could be readily screened for infectious disease therapy. In addition, chemical families of efflux pump inhibitors, specifically targeting active transport in the bacterial cell, have been described and characterized [134]. Among them, several inhibitor compounds, such as arylpiperidines [120], demonstrate efficient blocking of the efflux pump activity involved in the MDR phenotype as observed in many gram-negative clinical isolates [134].

Given the continuing emergence of MDR pathogens, the need for new antibiotics is acute and growing. The antibiotic drug discovery pipeline may be supported by creative approaches based on the understanding of antibiotic-resistant molecular mechanisms. Therefore, resistance gene products, their origins, evolution, distribution throughout bacterial populations and mode of action may provide new insights for the development of alternative strategies having a significant impact on the treatment of infectious diseases.

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5
Fitness Costs of Antibiotic Resistance

Pietro Alifano

5.1
Introduction

Antibiotics target essential microbial functions. Therefore, it is not surprising that newly acquired antibiotic resistances often impose fitness costs, which result from disturbance of cellular functions and enzymes and are usually expressed as reduced growth rates in antibiotic-free environments. For example, some point mutations in the *rpsL* gene in *Escherichia coli* confer resistance to high concentrations of streptomycin, but reduce the Darwinian fitness of bacteria by decreasing peptide chain elongation rates [1], while the acquisition of a plasmid-containing antibiotic-resistance genes by horizontal gene transfer (HGT) may reduce the growth rate due to the extra burden of DNA replication and gene expression [2].

The presence of such costs predicts that if antibiotic use was reduced, antibiotic-resistance frequency would decrease because the more fit susceptible bacteria would outcompete the resistant ones [3]. In fact, reduction in the use of antibiotics has been proposed as a measure to forestall, and ideally reverse, the growing public health problem of antibiotic resistance. This recommendation is supported by well-described correlations between the frequency of acquired resistance in targeted bacterial populations and the consumption of antimicrobial drugs [4–6].

However, in spite of these correlations, molecular epidemiological studies monitoring the temporal changes in the frequency of resistance to a specific antimicrobial drug when the drug consumption is deliberately reduced have yielded conflicting results. On one hand, a 50% reduction in the frequency of macrolide-resistance group A streptococci (from 16.5% in 1992 to 8.6% in 1996) was reported in Finland following reduced consumption of macrolides [7]. Similar successful interventions were reported in Iceland and in France to reduce the frequency of penicillin nonsusceptible *Streptococcus pneumoniae* (PNSP) [8, 9].

On the other hand, several studies demonstrate failure of such interventions. For instance, data published in 2001 demonstrate that a 98% decrease in sulfonamide prescriptions during the 1990s in the United Kingdom was followed by a 6.2% increase in the frequency of sulfonamide-resistant *E. coli*; sulfonamide resistance persisted undiminished 5 years later [10]. Interestingly, higher rates of PNSP in

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Jewish than in Bedouin community children, despite significantly lower prescription rates for penicillins, were associated with significantly higher prescription rates for azithromycin, supporting the idea that use of long-acting macrolides was an important factor in increasing penicillin resistance in a given community [11].

Altogether, these finding indicate that although there is a clear association between heavy antimicrobial consumption within a population and the frequent recovery of resistant bacteria, whether a reduction in antimicrobial use can reverse this process is less clear. Many recent studies clearly demonstrate that the fate of chromosomal and transposon- and/or plasmid-borne resistance determinants, following a reduction in the selective pressure, depends on factors other than drug consumption alone. An off-putting view based on theoretical arguments, mathematical modeling, experiments, and clinical interventions suggests that the resistance problem we have generated during the past 60 years because of the extensive use and misuse of antibiotics is here to stay for the foreseeable future [12]. These considerations emphasize the importance of quantifying the fitness costs associated with antibiotic resistance to predict the dynamics of the evolution of resistance.

The scope of this chapter is to concisely describe (i) available methods and mathematical models to estimate the fitness cost of an antibiotic-resistance determinant and to predict its fate, (ii) factors affecting the fitness cost of and antibiotic resistance other than the presence of the specific antibiotic, and (iii) mechanisms and dynamics causing persistence of chromosomal and plasmid-borne resistance determinants.

5.2
Methods to Estimate Fitness

Defining the effects of drug resistance on relative fitness can be difficult. Indeed, microbial fitness is by itself a complex trait that encompasses the ability of a given strain to survive and reproduce in a given environment. Furthermore, for commensal, opportunistic, or pathogenic microorganisms, it is also affected by host-to-host transmission capabilities. Different approaches and mathematical models are commonly used to estimate this trait, including experimental methods and epidemiological studies. No one method is likely to be sufficient to define it because fitness is dependent on multiple biological properties, and so multiple approaches and mathematical models are required.

5.2.1
Experimental Methods

Growth rate and generation time are accepted measures of fitness deficit associated with antibiotic resistance by using resistant and susceptible strains in pure culture or in pair competition assays, where two strains of interest are mixed together in equal proportion and left to compete head-to-head in a common environment

[13]. In addition to growth rate and generation time, other parameters can be used to measure fitness including quantification of biofilm and growth/survival under stressful conditions [14]. However, as antibiotics target essential physiological and biochemical functions, the fitness costs of resistance will depend on both physical and chemical growth conditions *in vitro*.

The methods based on cultivation of microorganisms *in vitro* (in growth media) rely on the assumption that what is true *in vitro* is also true *in vivo*, and that *in vitro* environments, although not faithful replicas of the *in vivo* environments, allow the dissection and analysis of biological phenomena in an easy and repeatable manner. This assumption is supported by the practical usefulness of these methods in addressing key aspects of microbial genetics and metabolism. However, it has become clear that the *in vitro* methods are not adequate to analyze complex traits including fitness, which depend on the interactions between microbes and specific environments.

For microorganisms living on animal hosts, competitive fitness may also be evaluated in animal models, such as a coinfected mouse. Results obtained *in vitro* (in growth media) and *in vivo* (in animal models) may be very different: resistant mutants that do not exhibit a fitness cost when tested in growth media may show a large cost in animal models, and vice versa [15]. There is also evidence that the process of adaptation to the costs of antibiotic resistance by secondary mutations that compensate for the loss of fitness without reducing the level of resistance may be very different in growth media and animal hosts [16], implying that making predictions about the evolution of antibiotic-resistant pathogens is difficult without *in vivo* experimentation.

5.2.2
Epidemiological Methods

For pathogenic microorganisms, epidemiological methods may be used to estimate the fitness burden associated with drug resistance. Darwinian fitness is defined as ''the likelihood to survive and reproduce.'' In pathogenic microorganisms, a complex interplay between ''infectiousness,'' ''transmissibility,'' and ''virulence'' determines this trait. Therefore, in infectious disease epidemiology, the absolute number of secondary cases generated (also known as the basic reproductive rate, R_0) represents the measure that reflects the absolute fitness of a pathogen. In addition to the absolute fitness, an often more useful measure is that of ''relative fitness,'' which compares the success of a particular pathogen variant (for example, a drug-susceptible strain) to the success of another (e.g., a drug-susceptible strain).

Evaluation of relative fitness associated with antibiotic resistance may be inferred by using odds ratios from molecular epidemiology data that allow classification of isolates into genotypic classes (clusters). The relative fitness of resistant strains compared with that of sensitive strains can be quantified from comparison of their genetic clustering. A cluster is defined as a group of cases in a community, which are caused by isolates that share similar or identical genotypes (or DNA fingerprinting) and are therefore epidemiologically linked.

These isolates represent cases of active disease transmission. In contrast, strains with distinct or unique DNA patterns are believed to reflect cases of reactivation of latent infections. The relative proportion of genotype clustering in drug-resistant and drug-sensitive strains can be transformed into a measure equivalent to the relative fitness and used to measure spreading of single or multiple antibiotic resistance. The use of genetic clustering in determining fitness is, however, considered an indirect method that does not take into account the dynamics of disease transmission, evolution of resistance, and mutation of molecular markers accounting for discrepancies observed in many studies. These limitations may be overtaken or blunted by mathematically modeling and applying robust statistic methods such as a recently proposed form of Bayesian computation [17].

5.3 Factors Affecting Fitness

5.3.1 Genetic Nature of the Resistant Determinant

The relative fitness of antibiotic-resistant strains can be influenced by the genetic nature of the resistance determinant. In particular, for chromosomally encoded resistance determinants, the specific antibiotic-resistance-conferring mutation affects the likelihood of surviving and reproducing under a variety of growth conditions.

The property of several resistance determinants to reduce the relative fitness and to attenuate bacterial virulence in animal models has been known for a long time. In 1963, Falkow and coworkers found that streptomycin-resistant (Str^R) mutants of *Shigella flexneri*, which required the presence of streptomycin for optimal growth, were avirulent for the guinea pig. Many years later, it became apparent, however, that Str^R-conferring mutations varied in their effects on bacterial fitness [1, 18].

Acquired resistance to high concentrations of streptomycin is usually the result of some point mutations in the *rpsL* gene coding for ribosomal small subunit protein S12, and the fitness burden of the Str^R mutants is mostly caused by

Table 5.1 Fitness costs and peptide chain elongation rates of Str^R mutations affecting the nucleotide sequence of *rpsL* gene in *Escherichia coli*.

rpsL	DNA sequence at codon 42	Cost of resistance (% per generation ± 1 standard error)	Peptide chain elongation rate (amino acid per second, ± 1 standard error)
Wild type	AAA	—	18.26 ± 1.41
K42T	ACA	13.6 ± 0.57	10.60 ± 0.70
K42N	AAC	18.8 ± 0.79	8.74 ± 0.56

Source: Data from Ref. [1].

decreased peptide chain elongation rates [1]. Studies in *E. coli* and *Salmonella enterica* demonstrated that the cost of resistance as well as the effect on peptide chain elongation rate varies depending on the nature of mutation (Table 5.1, Table 5.2, and Table 5.3).

The Str^R phenotype can be subdivided into two major groups: restrictive and nonrestrictive [19]. The restrictive bacteria have a characteristically lower frequency of nonsense suppression *in vivo*, and are also slower than the wild type in their rate of protein synthesis. Str^R mutations affecting the DNA sequence at codon 42 (AAA) specifying a Lys residue in the wild-type *rpsL* gene of *E. coli* and S. *enterica* may exhibit either the restrictive or nonrestrictive phenotype. All these mutations confer high levels of resistance to streptomycin but are known to affect bacterial fitness and virulence to different extent. The nonrestrictive Lys to Arg mutation (K42R) is a no-cost resistance mutation that does not affect fitness, while the restrictive Lys to Asn (K42N) and Lys to Thr (K42T) mutations have significant effects on fitness.

Table 5.2 Generation times, UGA suppression, and virulence of *Salmonella enterica* sv. Typhimurium strain LT2 Str^R mutants.

rpsL	DNA sequence at codon 42	Generation time in M9 glucose medium (min)	UGA 189 suppression[a]	UGA 220 suppression[a]	Virulence in mice[b]
Wild type	AAA	47	16	81	1
K42T	ACA	56	2	12	0.01
K42N	AAC	62	2	12	0.001

[a] UGA189 and UGA220 show the read-through of each nonsense mutation at that position in the *lacI* part of a *lacIZ* fusion, expressed as suppression $\times 10^4$.
[b] Virulence is measured by competition against wild-type LT2 in mice beginning with equal numbers of each cell type. The wild-type virulence value is set at 1. All virulence values are the proportion of mutant cells present in the cell population 4 days after infection.
Source: Data from Ref. [19].

Table 5.3 Relative fitness of *Salmonella enterica* sv. Typhimurium strain LT2 Str^R mutants in mice and in lysogeny broth (LB) medium.

rpsL	DNA sequence at codon 42	Relative fitness in mice[a]	Relative fitness in LB[a]
Wild type	AAA	1.00	1.00
K42N	AAC	0.50	0.79
K42R	AGA	1.00	0.96

[a] Relative fitness is defined as the generation time of the wild type divided by the generation time of the mutant.
Source: Data from Ref. [16].

Similar conclusions were drawn from an experimental study with *Mycobacterium smegmatis* (Table 5.4), which also demonstrated that the *rpsL* mutations associated with no-cost or with the least fitness cost were the most frequent in clinical isolates of *Mycobacterium tuberculosis* [20]. It is worth noticing that in *M. tuberculosis*, owing to the presence of a single *rrn* operon, acquired resistance to streptomycin is often caused by point mutations affecting the 16S rRNA encoding gene. Also, in

Table 5.4 Fitness costs of Str^R mutations affecting the nucleotide sequence of *rpsL* gene in *Mycobacterium smegmatis*.

rpsL	DNA sequence at codon 42	Cost per generation in *b*rain heart infusion (BHI) medium (%)	Relative fitness in BHI medium (95% confidence interval)
Wild type	AAA	—	100.0
K42T	ACA	14.98	76.4–91.1
K42N	AAC	14.10	79.9–89.6
K42R	AGA	0.99	95.8–102.1

Source: Data from Ref. [20].

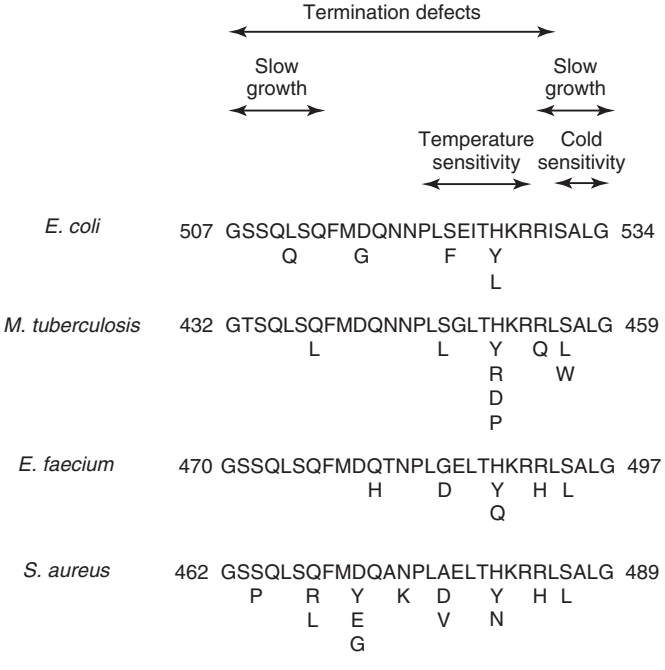

Figure 5.1 Structure of the rifampicin-resistance cluster I of the *rpoB* gene of *Escherichia coli*, *Mycobacterium tuberculosis*, *Enterobacter faecium*, and *Staphylococcus aureus*, showing the positions of individual mutations and, for *E. coli*, the associated phenotypes.

this case, an inverse correlation between the fitness cost associated with the *rrn* mutations and its frequency in clinical isolates was observed [20]. Altogether, these findings suggest that in clinical settings there is a strong selection pressure for drug-resistance-conferring mutations that cause minimal fitness defects.

Variable effects on bacterial fitness ranging from no cost to high cost have also been found to be associated with different mutations conferring resistance to rifampicin. Rifampicin binds to bacterial RNA polymerase and prevents productive initiation of transcription, but does not inhibit transcription after promoter clearance. Most of the mutations conferring rifampicin resistance (Rif^R) are clustered within three distinct sites, clusters I, II, and III (Figure 5.1), in the central segment of the β- chain of the RNA polymerase [18]. As these mutations, which change amino acids directly involved in antibiotic binding to RNA polymerase (Figure 5.2) [21], affect evolutionarily conserved residues, they are expected to compromise transcription efficiency and hence physiology and fitness of the organism. Indeed, a direct relationship between the fitness cost of *rpoB* mutations and their effects on transcription was demonstrated in *E. coli* (Table 5.5) [22]. In particular, Rif^R RNA polymerases have altered properties in transcription elongation and/or termination, and several Rif^R mutations are allele-specific suppressors of defective *nusA* and *rho* alleles [18]. In contrast, no obvious association between the magnitude of Rif^R and its allied cost was ever found in *E. coli* [23] as well as in other microorganisms (Table 5.5 and Table 5.6) [24].

However, a fitness burden is not always associated with *rpoB* mutations. For instance, the substitution D516G conferring intermediate resistance to rifampicin

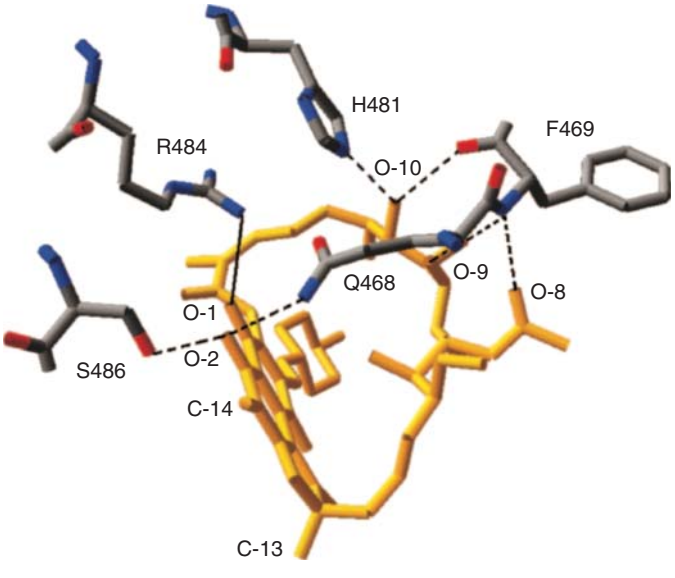

Figure 5.2 Model of rifampin (gold) binding to important residues in the *S. aureus* wild-type β-subunit RNA polymerase. *Source*: Figure reproduced with permission from Ref. [21].

Table 5.5 Fitness costs Rif^R mutations affecting the nucleotide sequence of the *rif* cluster I in the *rpoB* gene of *Escherichia coli*.

rpoB	MIC ($\mu g\ ml^{-1}$ rifampicin)[a]	Relative fitness: % growth/generation (vs K12 parent) ± SEM[b]	Transcription efficiency ± SEM[c]
Wild type	0–12.5	100 ± 0.1	0.058 ± 0.008
L511Q	25–50	86.5 ± 1.8	0.021 ± 0.001
D516G	100–200	103.0 ± 0.2	0.059 ± 0.009
H526Y	200–400	91.3 ± 1.0	ND
H526L	100–200	94.4 ± 1.5	ND

[a]The concentration interval indicated for MIC denotes the range within which the true MIC for rifampin exists.
[b]Relative fitness (mutation cost) was determined via direct competition between Rif^r mutants and a Rif^s K12 MG1655. The standard error for the cost estimate is shown in parentheses. A value below 100% indicates that the strain tested was at a reproductive disadvantage relative to the wild-type reference strain. A fitness value in excess of 100% indicates that the strain tested exhibited a reproductive advantage relative to the wild type.
[c]Transcription efficiency was examined using a semiquantitative RT-PCR assay. This assay measured the kinetics of production of a full-length induced transcript, lactose transacetylase (*lacA*; the 3′-most mRNA encoded on the *lac* operon), relative to that of an internal steady-state control, *recA*, as a function of time postinduction. ND, not determined.
Source: Data from Ref. [22].

Table 5.6 Fitness costs Rif^R mutations affecting the nucleotide sequence of the *rif* cluster I in the *rpoB* gene of *Staphylococcus aureus*.

rpoB	MIC ($\mu g\ ml^{-1}$ rifampicin)	Relative fitness (mean ± SEM)[a]
Wild type	≤0.008	1
S464P	4	0.93 ± 0.008
Q468R	>512	0.80 ± 0.007
Q468L	512	0.95 ± 0.016
D471Y	4	0.88 ± 0.012
D471E	0.5	0.96 ± 0.008
D471G	0.5	0.87 ± 0.022
N474K	8	0.60 ± 0.020
A477D	256	0.91 ± 0.013
A477V	1	0.88 ± 0.023
H481Y	512	0.93 ± 0.011
H481N	4	1 ± 0.004
R484H	256	0.75 ± 0.019
S486L	512	0.86 ± 0.011

[a]The relative fitness of the Rif^S parental strain and the Rif^R mutants were determined by paired competition experiments from the ratio of the number of generation from Rif^R to Rif^S strain.
Source: Data from Ref. [24].

was instead associated to a slight fitness advantage in *E. coli* [22] (Table 5.5). Substitution of the conserved histidine residue in the cluster I of *rpoB* is extremely frequent among clinical Rif^R isolates in many bacterial species, reflecting the low fitness cost imposed by amino acid substitutions at this position. In fact, the substitution H481N in the *rpoB* gene of *Staphylococcus aureus* was not demonstrably associated with a cost of resistance *in vitro* [24] (Table 5.6). In contrast, the substitution H526Y in *E. coli* and the corresponding substitution H481Y in *S. aureus* (Table 5.6) gave an appreciable, although modest, fitness burden [24].

Molecular modeling has adequately explained the major cost associated with the substitution H481Y with respect to that of the substitution H481N in *S. aureus* [21]. Substitution of the imidazole ring in histidine 481 for the phenolic moiety in tyrosine results in hydrogen bonding between tyrosyl hydroxyl group and the proximal guanidine moiety of the arginine 484 (Figure 5.2). As the arginine 484 lies at the surface of RNA polymerase and is predicted to be in contact with DNA, the hydrogen bonding would move the arginine residue away from its original position, thus weakening the electrostatic interaction with the DNA template and decreasing the stability of the transcription complex.

The fitness burden of a given substitution may also vary between different species. In *M. tuberculosis*, the *rpoB* S531L mutation, which is the most frequent Rif^R-conferring mutation in clinical strains worldwide, was associated with the lowest fitness cost in laboratory strains and no fitness defect in clinical strains [25]. However, in *S. aureus*, the corresponding substitution S486L significantly affected bacterial growth rates [24] (Table 5.6).

While many studies have investigated the effects of chromosomal antibiotic-resistance-conferring mutations on bacterial fitness and the mechanisms alleviating the fitness burden (see subsequent text), fewer studies have examined fitness costs associated with acquired transposon- and/or plasmid-borne antibiotic-resistance genes. In general, resistance plasmids impose an initial fitness cost on their hosts [2]. However, *in vitro* studies performed in *E. coli* with pBR322 [26], pACYC184 [27], R1, and RP4 [28] have demonstrated that, after a period of coevolution, compensatory mutations can arise, with the plasmid-carrying host becoming fitter than its plasmid-free derivative. Similar results were also obtained with plasmid R46 both *in vitro* and in a pig gut *in vivo* model [29]. The only study that experimentally investigated the biological cost of a *vanA* plasmid in *Enterococcus faecium* conferring resistance to glycopeptides antibiotics reported a 4% reduced fitness relative to the plasmid-free ancestor *in vitro* and in gnotobiotic mice [30] accounting for rapid decline in glycopeptides resistant *E. faecium* occurrence following the ban on avoparcin.

Competitive *in vitro* assays have shown variable fitness costs associated with acquisition of transposons encoding antibiotic-resistant genes. The kanamycin-resistance transposon Tn*5* has been reported to confer a selective advantage on *E. coli* owing to the presence of the bleomycin-resistance gene *ble*, the product of which is able to prevent DNA breakage [31]. In contrast, Tn*10* acquisition was found to be associated with a fitness cost *in vitro*. This cost was approximately equal regardless of whether the transposon encoded tetracycline, chloramphenicol,

or kanamycin resistance and was thought to be due to insertion mutations [23]. Acquisition of Tn*7* had no impact on the fitness of *E. coli* both *in vitro* and in the pig gut model, while acquisition of Tn*1* improved fitness in the case of a first derivative, but in the case of a second, independent derivative, Tn*1* had a neutral effect on fitness [29].

5.3.2 Expression of the Antibiotic-Resistance Determinant

Appropriate gene regulation has been shown to reduce or eliminate the fitness cost of an antibiotic-resistance determinant in the absence of direct antibiotic selection. A clear example is provided by a study on VanB-type vancomycin resistance in *E. faecium* and *Enterococcus faecalis* [32]. Acquired VanA- and VanB-type resistance to the glycopeptides vancomycin and teicoplanin in enterococci is due to the synthesis of modified peptidoglycan precursors ending in D-alanyl-D-lactate (D-Ala-D-Lac), to which glycopeptides exhibit low binding affinities, together with elimination of the high-affinity D-Ala-D-Ala ending precursors [33]. As in VanA-type strains, in VanB-type strains, synthesis of D-Ala-D-Lac requires a dehydrogenase ($VanH_B$) that converts pyruvate to D-Lac and a ligase (VanB) of altered specificity compared with the host D-Ala:D-Ala ligase (Ddl). Removal of precursors terminating in D-Ala is catalyzed by a D,D-dipeptidase ($VanX_B$) and a D,D-carboxypeptidase ($VanY_B$) [33].

While the *vanA* gene cluster in VanA-type strains is part of transposon Tn*1546*, which is often carried by self-transferable plasmids [30], the VanB-type resistance is associated with the conjugative transposon Tn*1549*. In VanB-type strains, expression of resistance is induced by vancomycin and regulated by a two-component regulatory system composed of a sensor ($VanS_B$) and a regulator ($VanR_B$) that acts like a transcriptional activator. Induction of the sensor leads to expression of the regulatory ($vanR_BS_B$) and resistance ($vanH_BBX_B$) operons [34]. Mutations in $vanS_B$ leading to constitutive expression of resistance have been obtained *in vitro* and *in vivo* but are rare in clinical settings.

Tight regulation of resistance expression drastically reduces the biological cost associated with vancomycin resistance in VanB-type *E. faecium* and *E. faecalis*, and accounts for the widespread dissemination of these strains. The study of Foucault and coworkers [32] demonstrates that both *in vitro* and *in vivo* using gnotobiotic mice carriage of inactivated or inducible Tn*1549* had no cost for the host in the absence of induction by vancomycin, while, in contrast, induced or constitutively resistant strains not only had reduced fitness but were severely impaired in colonization ability and dissemination among mice. These findings also suggest that the 4% reduction in fitness, which was observed in a VanA-type *E. faecium* strain by comparing the *in vitro* competitiveness of the resistant strain harboring a *vanA* plasmid with that of its plasmid-free counterpart, was more likely due to the cost of carrying a large-sized (>100 kb) plasmid than to metabolic burden.

5.3.3 Microbial Cell Physiology, Metabolism, and Lifestyle

As antibiotics target essential functions, the fitness costs of resistance will depend on microbial cell physiology, metabolism, and lifestyle. As a consequence, the fitness burden, as well as the susceptibility to antimicrobial drug, may vary under different growth conditions. Resistant mutants that fail to show fitness cost *in vitro* may have a large cost in animal models, and vice versa [16]. Also, *in vitro*, the fitness may vary greatly depending on the growth medium.

We have previously seen that the Str^R mutations K42N and P90S in the ribosomal protein S12 impair growth on rich medium. Surprisingly, in media with poorer carbon sources, these same mutations confer a selective advantage, allowing the Str^R mutant strains to grow faster than the wild type [35]. The improved growth reflects a failure of these Str^R mutants to induce the stress-inducible sigma factor RpoS (σ^S), a key regulator of many stationary-phase and stress-inducible genes. On poorer carbon sources, wild-type cells induce σ^S, which retards growth. By not inducing σ^S, Str^R mutants escape this self-imposed inhibition. Indeed, the Str^R mutant loses its advantage over the wild type when both strains lack the σ^S-encoding gene. This finding also provides an alternative explanation for the avirulence of the K42N mutant. It was previously suggested that the low virulence of this mutant is a direct consequence of the reduced polypeptide elongation rate and associated reduction in growth rate (see preceding text). However, it is possible that the disturbed induction of the σ^S in the mutant and the resulting poor induction of σ^S-regulated virulence gene may contribute to the reduction in virulence.

Also, Rif^R mutations affecting the RNA polymerase structure may be conditionally beneficial depending on the carbon source substrate. For instance, it has been demonstrated that Rif^R *rpoB* mutants of *Bacillus subtilis* can present novel metabolic capabilities with fitness gain when compared with their rifampicin-susceptible parental strain [36]. The resistant mutants make less proficient use of strongly utilized substrates, but increase their capability to degrade weakly utilized substrates. Interestingly, different Rif^R mutations have different effects on the carbon source metabolism likely because the interactions of RNA polymerase with the different promoters change depending on the mutation involved [36].

A similar effect of antibiotic resistance was observed in a *Stenotrophomonas maltophilia* mutant selected by antibiotic pressure, which overexpresses the MDR (multidrug-resistant) efflux pump SmeDEF [37]. This strain is more proficient than its wild-type counterpart in the utilization of sugars such as gentibiose, dextrin, and mannose, as well as formic acid. In contrast, the antibiotic-resistant mutant was impaired in the utilization of amino acids such as alanine, serine, or proline [38]. This result indicates that antibiotic resistance due to SmeDEF overexpression is associated with a ''metabolic shift'' more than a ''general metabolic burden'' in *S. maltophilia*.

This conclusion is supported by the result of a study with a *Pseudomonas aeruginosa* antibiotic-resistance mutant, which overexpresses the MDR efflux pump

MexCD-OprJ [39]. Proteomic analyses demonstrated that several proteins were differentially expressed in the mutant as compared with its wild-type isogenic parental strain. Among them, many played a role in amino acid and energy metabolism. The analysis of secreted metabolites showed that the resistant strain secreted higher levels of fatty acids such as myristic, palmitic, and stearic acids, which are major components of *P. aeruginosa* membranes.

These examples briefly illustrate how antibiotic-resistance determinants may profoundly affect bacterial physiology. These physiological changes include specific alterations in bacterial metabolism that can even be adaptive for colonizing specific ecosystems, highlighting the importance of measuring fitness costs under multiple experimental conditions. When growth media are used, both the sign and the magnitude of any fitness effect may be affected by physical and chemical parameters, including nutrient source, pH, redox, and salt conditions [40]. Preferably, fitness costs should be measured under conditions as close to *in vivo* as possible.

5.3.4 Genetic Background of the Antibiotic-Resistant Mutant

As the fitness burden of antibiotic resistance is intrinsically linked to bacterial physiology, metabolism, and lifestyle, it is not surprising that it is also influenced by the genetic context (i.e., strain background). For example, experiments in a chicken infection model with *Campylobacter jejuni* demonstrated that a specific quinolone-resistance-conferring mutation in the DNA gyrase gene *gyrA* reduced the relative fitness of some quinolone-resistant strains, but increased strain fitness when transferred into another strain background [41].

Similar conclusions were drawn from experimental studies with isoniazid-resistant strains of *M. tuberculosis*. The different lineages of this pathogen differ in immunogenicity and virulence in animal models, and influence the outcome of infection and disease in humans [42]. Moreover, there is evidence that the variable genetic background of strains belonging to the different lineages could play a role in the evolution of drug resistance.

In particular, the Beijing lineage has repeatedly been associated with drug resistance. A study looking at the *in vitro* growth of clinical strains demonstrated that, in contrast to non-Beijing strains, some drug-resistant strains belonging to the Beijing lineage had no growth defect compared to their drug-susceptible counterparts [43]. Furthermore, a study in San Francisco showed that Beijing strains were significantly associated with isoniazid-resistance-conferring mutations that were likely to abrogate *katG*-encoded catalase/peroxidase activity. As previously shown, this activity helps protect the bacteria against oxidative stress during infection, and hence loss of *katG* usually results in attenuation [44]. The high prevalence of isoniazid resistance among Beijing strains suggests that bacteria belonging to this lineage might be less dependent on an intact *katG*, perhaps because they are generally less susceptible to oxidative stress or better able to compensate for the loss of *katG* activity.

5.4
Mechanisms and Dynamics Causing Persistence of Chromosomal and Plasmid-Borne Resistance Determinants

We have experienced that on restricted use of antibiotics, rates of antibiotic resistance usually fall but do not vanish, and stable rates of resistance in the apparent absence of direct selection pressure persist. This persistence may be due to either low-level antibiotic contamination that maintains the selective pressure or to the stability of the antibiotic-resistant determinant. Several processes are known to reduce the reversal of acquired antimicrobial drug resistance in the absence of the corresponding drug including (i) low cost or no cost of antimicrobial-resistance determinant, as previously discussed; (ii) compensatory genetic mechanisms that restore or improve fitness without loss of resistance; (iii) linked selection and segregational stability of resistance determinant; and (iv) reacquisition of antimicrobial resistance.

5.4.1
Compensatory Genetic Mechanisms That Restore or Improve Fitness without Loss of Resistance

The fitness burden of antibiotic resistance can be reversed, at least partially, by compensatory mechanisms, often without reducing the level of resistance [45]. These mechanisms include (i) point mutations within or outside the resistance gene, (ii) gene amplification, (iii) gene duplication, and (iv) gene conversion.

The mechanisms responsible for adaptation to the fitness costs imposed by chromosomally encoded resistance have been studied in detail in *E. coli* [1, 46] and *S. enterica* [16, 19]. In particular, for *S. enterica* Str^R *rpsL* mutants grown in a laboratory medium, fitness improvement is mainly achieved via compensatory mutations in ribosomal proteins encoded by *rpsD* (encoding ribosomal protein S4) and *rpsE* (encoding ribosomal protein S5). Such mutations foster restoration of protein elongation rates. Notably, in a mice model of infection, amelioration of the cost of *rpsL* mutations is principally obtained via intragenic mutations (for instance, the R93H substitution that compensates the K42T) or intracodonic single or double mutations resulting in replacement of restrictive *rpsL* alleles with nonrestrictive ones [16] (Table 5.7). These studies demonstrate that compensatory mutations are more common than reversion to the sensitive phenotype and that the result of evolution in an antibiotic-free environment may be completely different *in vitro* or *in vivo*. In general terms, the rates and directions of molecular evolution may follow different trajectories because of the specific environment and its influence on mutation formation or selection.

Similar conclusions were drawn from fusidic-acid-resistant (Fus^R) mutants of the same microorganism [16]. Fus^R is caused by mutations in the *fusA* gene coding for translation elongation factor G (EF-G). Resistant mutants grow slowly in laboratory media as a consequence of a decreased rate of protein synthesis. After serial passage in a laboratory medium in the absence of antibiotic, spontaneous

Table 5.7 Fitness in mice and in LB medium of Str^R and compensated *Salmonella enterica* sv. Typhimurium mutants.

Strain	Mutation		Compensated selected conditions[a]	Relative fitness in mice[b]	Relative fitness in LB medium
	Ribosomal protein S4	Ribosomal protein S12			
JB124	wt	wt	NA	1.0	1.0
JB127	wt	K42N (AAC)	NA	0.50	0.79
JB2162	Q53L	wt	NA	0.62	0.68
TH5461	Q53L	K42N	LB	1.0	0.93
TH5664	K205N	K42N	LB	0.94	0.81
TH5604	Q53P	K42N	LB	0.91	0.96
TH5606	V200	K42N	LB	0.91	0.90
TH5516	I199N	K42N	LB	0.91	0.90
TH5667	UAG201	K42N	LB	0.91	0.84
JB1258	wt	K42R (AGA)	Mice	1.0	0.96

wt, Wild type; NA, not applicable.
[a] Growth conditions under which the compensated mutants were selected.
[b] Relative fitness is defined as the generation time of the wild type divided by the generation time of the mutant.
Source: Data from Ref. [16].

mutants are selected by virtue of their faster growth rates. Most of the compensatory mutations are located within *fusA*. However, while serial passage in a laboratory medium resulted in outgrowth of intragenically compensated mutants, evolution in mice resulted almost exclusively in reversion at the *fusR* (*fusA*) locus (Table 5.8). This behavior has been justified by taking into account that *fusR* (*fusA*) mutants have altered levels of (p)ppGpp, a pleiotropic regulator of gene expression. Altered concentrations of (p)ppGpp could affect the expression of virulence-related genes, resulting in a significant fitness defect in mice, without necessarily affecting growth in a laboratory medium [16].

Compensatory mutations were also much more frequent than reversion to drug sensitivity in Rif^R (*rpoB*) *E. coli* mutants, which evolved to become more fit than their ancestors in a laboratory medium for 200 generations both in the presence and in the absence of rifampicin [22] (Table 5.9). In nearly all cases, gains in fitness were coincident with improved transcription efficiency. Interestingly, in the evolution experiments in the presence of rifampicin, overall levels of resistance increased as did relative fitness, leading to the belief that the combination of sublethal drug exposure and intermediate- or low-level resistance may have unfortunate consequences in long-term clinical care [22]. In this context, the D516G substitution in *rpoB*, which arose under conditions of selection for enhanced resistance to a Rif^R strain harboring the L511Q substitution, is of particular interest because the evolved double L511Q + D516G mutant exhibited fitness either greater than

Table 5.8 Fitness in mice and in LB medium of Fus^R and compensated *Salmonella enterica* sv. Typhimurium mutants.

Strain	Mutation (number of independent isolates found)	Compensated selected conditions[a]	Relative fitness in mice[b]	Relative fitness in LB medium
JB124	Wt	NA	1.0	1.0
JB393	P413L	NA	No growth	0.41
JB2080	wt (Revertant) (2)	LB	1.0	1.0
JB2124	P413L, G13C (3)	LB	0.94	1.0
JB2111	P413L, L413Q (3)	LB	0.85	1.0
JB2105	P413L, R407G (1)	LB	0.82	0.90
JB2117	P413L, A378V (3)	LB	0.81	1.0
JB2115	P413L, G13A (1)	LB	0.79	1.0
JB2108	P413L, V363F (1)	LB	0.74	0.96
JB2119	P413L, L413V (1)	LB	0.68	1.0
JB2104	P413L, A66V (2)	LB	0.66	1.0
JB2112	P413L, I294S (1)	LB	0.64	1.0
JB2122	P413L, V376A (3)	LB	0.63	1.0
JB2114	P413L, F444L (3)	LB	0.59	0.96
JB2109	P413L, A378T (1)	LB	0.54	0.87
JB2113	P413L, L387P (1)	LB	0.42	0.93
JB2120	P413L, V291E (1)	LB	0.36	1.0
JB2110	P413L, T423I (1)	LB	0.33	0.90
JB2153	wt (Revertant) (14)	Mice	1.0	1.0
JB2180	P413L, F334L (7)	Mice	0.72	ND
JB1777	P413L, I294D (3)	Mice	0.52	1.0
JB1744	P413L, P683L (1)	Mice	0.29	0.96

wt, wild type; NA, not applicable; ND, not determined.
[a]Growth conditions under which the compensated mutants were selected.
[b]Relative fitness is defined as the generation time of the wild type divided by the generation time of the mutant.
Source: Data from Ref. [16].

or roughly equal to either single mutant (or the wild type), and much higher resistance to rifampicin [22] (Table 5.9). Noticeably, the L511Q + D516G double substitution has been found many times in independent clinical Rif^R isolates of *M. tuberculosis* [47].

However, there is strong evidence that intergenic compensation contributes much more than intragenic compensation to the emergence of MDR Rif^R *M. tuberculosis* strains in human populations [48]. Whole-genomic comparison of 10 paired clinical strains (Rif^R isolates and Rif^S isolates, which were recovered from the same infected individuals at different time points) and 6 *in vitro*-evolved Rif^R strains demonstrated that the acquisition over time of particular mutations in *rpoA* and *rpoC* genes, coding for RNA polymerase α- and β′-chains, respectively, leads to the emergence of MDR strains with high fitness. *In silico* analysis indicates that the compensatory mutations are localized to the interface between α- and

Table 5.9 Characteristics of evolved Rif^R *E. coli* K12 (rif-1, rif-2, rif-8, rif-9).

Strain[a]	Relative fitness: % growth/ generation (vs mutant parent) (± SEM)	MIC ($\mu g\,ml^{-1}$ rifampicin)	Original *rpoB* substitution	Secondary *rpoB* substitution	Transcription efficiency (± SEM)
K12 (MG1655)	ND	0–12	NA	NA	0.058 (0.008)
rif-1	100	100–200	I572L	NA	0.029 (0.004)
E-rif-1A	119.9 (1.5)	100–200	I572L	None identified[b]	0.058 (0.014)
E-rif-1B	114.1 (1.3)	100–200	I572L	None identified	0.042 (0.009)
E-rif-1C	112.1 (0.7)	100–200	I572L	None identified	ND
E-rif-1D	114.2 (0.8)	100–200	I572L	None identified	ND
ER-rif-1A	117.1 (1.1)	400–800	I572L	D516G	0.051 (0.018)
rif-2	100	25–50	L511Q	NA	0.021 (0.001)
E-rif-2A	110.7 (1.5)	25–50	L511Q	None identified	0.067 (0.014)
E-rif-2B	110.8 (1.2)	25–50	L511Q	None identified	ND
E-rif-2C	105.9 (1.4)	25–50	L511Q	None identified	0.029 (0.002)
E-rif-2D	107.7 (1.9)	25–50	L511Q	None identified	ND
ER-rif-2A	111.0 (0.7)	800–1000	L511Q	D516G	0.060 (0.014)
ER-rif-2B	113.2 (1.9)	800–1000	L511Q	D516G	0.059 (0.009)
rif-8	100	3000–4000	P564L	NA	0.019 (0.003)
E-rif-8A	109.6 (0.6)	3000–4000	P564L	R211P	0.040 (0.001)
E-rif-8B	113.5 (1.0)	3000–4000	P564L	None identified	0.027 (0.009)
E-rif-8C	114.1 (0.7)	5000–6000	P564L	None identified	ND
E-rif-8D	115.6 (0.6)	5000–6000	P564L	S574F	0.031 (0.009)
ER-rif-8A	114.9 (3.0)	3000–4000	P564L	L194R	0.039 (0.019)
ER-rif-8B	115.9 (8.7)	5000–6000	P564L	S574F	0.044 (0.005)
rif-9	100	100–200	D516G	NA	0.059 (0.009)
E-rif-9A	97.5	400–800	D516G	S574Y	0.038 (0.003)
E-rif-9B	98.7	400–800	D516G	H554Y	0.047 (0.007)
E-rif-9C	98.6	400–800	D516G	S574Y	ND

[a] E strains were passaged without drug. ER strains were evolved under drug selection pressure ($25\,\mu g\,ml^{-1}$ rifampicin).
[b] G556G (GGT to GGG).
Source: Data from Ref. [22].

β′-subunits, suggesting that they potentially affect the interaction between these subunits (Figure 5.3) [48].

In vitro evolution to ameliorate the fitness burden of mupirocin resistance (Mup^R) provides further evidence of how drug-resistant bacteria may improve fitness while leaving resistance unaffected [15, 49]. Mupirocin is an analog of isoleucyl–adenylate and inhibits protein synthases by binding to class I isoleucyl-tRNA synthetase (IleRS), preventing attachment of isoleucine to its cognate tRNA [50]. Point mutations in the chromosomally encoded *ileS* gene were shown to confer low-level resistance [51], and were frequently found in Mup^R *S. aureus*

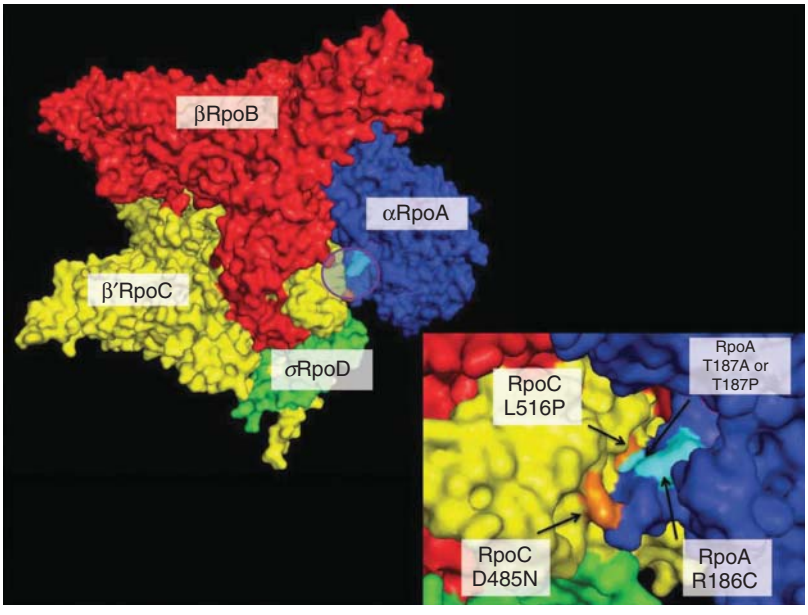

Figure 5.3 Rif^R compensatory mutations in *rpoA* and *rpoC* in regions. Amino acid substitutions identified in rifampicin-resistant experimentally evolved isolates and paired clinical isolates were mapped onto the structure of the *E. coli* RNA polymerase. The alterations are localized to residues of RpoA (light blue) and RpoC (orange) that are predicted to have roles in RNA polymerase subunit interaction. Residue numbers are indicated according to *M. tuberculosis* coordinates. RpoA (α subunit), blue; RpoB (β subunit), red; RpoC (β2 subunit), yellow; and RpoD (σ subunit), green. *Source*: Figure reproduced with permission from Ref. [48].

isolates from patients in long-term facilities [52]. Mup^R *ileS* mutations cause a severe reduction in fitness owing to impairment of the IleRS enzyme [15, 51]. *In vitro* evolution studies with Mup^R *S. enterica* demonstrated that the fitness burden could be alleviated by multiple mechanisms involving (i) secondary mutations in *ileS* restoring full activity of the enzyme leaving Mup^R, in most cases, unaffected; (ii) mutations in *ileS* promoter resulting in enhanced gene expression; and (iii) amplification of the *ileS* gene resulting in increased copy number [15]. In some adapted strains, a multistep process of adaptation initiated by gene amplification, followed by later acquisition of rare point mutations (*ileS* promoter and/or coding sequence) and, eventually, by loss of *ileS* extra copies seems to have occurred [49].

These studies demonstrate that the genetic flexibility associated with gene duplication and amplification events may be important because it increases the probability of getting rare mutations, as previously shown, and also because it can serve as a way of alleviating the cost of resistance or modulating it in response to antibiotic selection pressure. Advantages of these mechanisms are that they are frequent and reversible. The genetic mechanisms leading to fitness compensation in *S. enterica* mutants resistant to the peptide deformylase inhibitor actinonin provide further examples of how gene duplication and amplification events may

reduce the fitness burden of antibiotic resistance. These mutants carry mutations in either of two genes required for the formylation of methionyl initiator tRNA (tRNAi): *fmt* and *folD*. It has been shown experimentally that approximately one-third of the extragenically compensated *fmt* mutants carried amplifications of the tandemly repeated *metZ* and *metW* genes, encoding tRNAi. The increase in *metZ* and *metW* gene copy number was by up to 40-fold, increasing tRNAi levels and compensating for the lack of methionyl-tRNA formyltransferase activity [53].

Gene duplication and amplification events are also thought to alleviate the fitness cost associated with Rif^R mutations affecting the *rpoB* gene in several actinomycetes. In contrast to the widely accepted consensus of the existence of a single RNA polymerase in bacteria, actinomycetes with two or multiple *rpoB* paralogs were recently discovered [54, 55]. The presence of both wild-type *rpoB* (*rpoB[S]*) and a low-cost mutant Rif^R *rpoB* (*rpoB[R]*) allele in the same genome may represent an elaborate strategy to minimize the disadvantage associated with Rif^R. Furthermore, there is evidence that duplication of *rpoB* locus may have a regulatory significance. Indeed, expression of *rpoB(R)* is subject to upregulation during the late stage of the developmental life cycle of *Nonomuraea* sp. ATCC 39727 and constitutive expression of *rpoB(R)* lead to stimulation of secondary metabolism [54]. Moreover, when transferred to *S. lividans*, *rpoB(R)* activates cryptic antibiotic production, and there is evidence that the low-cost H426N (H526N in *E. coli*) *rpoB(R)*-associated mutation mimics (p)ppGpp binding to RNA polymerase [54].

Gene conversion involving paralogs is an additional mechanism that modulates bacterial fitness and antibiotic resistance levels. For example, in *S. aureus*, resistance to linezolid, which is caused by a mutation altering a 23S rRNA-encoding gene and is associated with a fitness cost, may be modulated, after removal or attenuation of the antibiotic selective pressure, by gene conversion between the multiple copies of the 23S rRNA-encoding gene where at least one copy had remained wild type in sequence [56]. A similar mechanism modulates bacterial fitness and antibiotic resistance levels in kirromycin-resistant bacteria. Kirromycin targets the translation elongation factor EF-Tu. Resistance level and fitness are strongly affected by gene conversion because many bacteria have duplicated EF-Tu-encoding *tufA* and *tufB* genes. Depending on the type of the selection force either for increased resistance or for increased fitness, either the resistance allele may be copied into the sensitive locus or vice versa [57].

5.4.2 Linked Selection and Segregation Stability of Resistance Determinants

Physical linkage between an antibiotic-resistance determinant and beneficial host genes (e.g., virulence genes or other antibiotic- or heavy metal-resistance genes) can favor persistence of the resistance determinant even in the absence of the antibiotic selective pressure. Coselection is a common feature of resistance that is acquired by HGT.

For example, the disappointingly small effect on trimethoprim resistance levels in *E. coli* following an intervention in Kronoberg County in Sweden, where the use

of trimethoprim-containing drugs was decreased by 85% was imputed to a combination of the small fitness cost measured for trimethoprim resistance together with a strong coselection for other antibiotics (e.g., mecillinam, furantoin, fluoroquinolones, cephalosporins, etc.), which remained in use during the intervention period [58]. Coselection with other resistant markers was also thought to contribute to failure of the above-mentioned intervention in Great Britain, aimed at reducing resistance to sulfonamides [10].

An example of linked selection between antibiotic-resistance and heavy metal-resistance determinants is represented by plasmid pUB101 of *S. aureus* harboring both fusidic acid and cadmium resistance genes. Selection of bacteria in the presence of high fusidic acid levels will simultaneously maintain cadmium resistance, even in the absence of cadmium and vice versa [59]. Analogously, a potential mechanism for persistence of plasmid-mediated VanA-type glycopeptides resistance in *E. faecium* in Danish poultry is represented by physical linkage between glycopeptides-resistance genes and both $CuSO_4$ and erythromycin-resistance determinants [60].

These literature examples confirm that the mechanisms governing the dynamics of an antibiotic-resistance determinant in the absence of antibiotic pressure in a bacterial population are many, and the fate of an unselected resistance marker is not easily predictable. For some human pathogens, expansion of hypervirulent and hyperepidemic clones has been conditioned by antibiotic pressure, which has played a role in restricting diversity during evolution and spread [61, 62]. The observed linkage between epidemicity and antibiotic resistance implies a physical linkage between resistance determinants and genes coding for host-to-host transmission, colonization and virulence factor, or immunological markers. Compelling examples are represented by a few successful clones of methicillin-resistant *S. aureus* [63] or clonal complexes of glycopeptide-resistant *E. faecium* [64], which have rapidly spread worldwide.

For plasmid-encoded antibiotic-resistance determinants, segregation stability is an additional factor contributing to persistence. Plasmid stability depends on multimer resolution, active partitioning, and postsegregation killing systems, which promote plasmid maintenance through selective killing of plasmid-free cells via a toxin–antitoxin mechanism. When the plasmid is lost, the bacterium is killed or inhibited as a result of the higher cytoplasmic stability of the toxin compared with the antitoxin. This mechanism contributes to the persistence of plasmid-encoded resistance in the absence of antimicrobial selection. Segregation stability of several plasmids harboring VanA-type glycopeptide-resistance determinants through toxin–antitoxin systems has been thought to affect long-term persistence in antibiotic-free environments [65, 66].

5.4.3
Reacquisition of Antimicrobial Resistance

The rate at which microorganisms reacquire resistance when the selective pressure of the antibiotic is relieved is critical in control of reversal of resistance. Bacteria

may reacquire resistance by spontaneous mutations or by HGT. In the first case, rates of spontaneous mutation are generally too low to undermine the reversal of resistance, although rates may be greatly elevated in mutator bacteria. Indeed, mutator phenotypes, mainly due to defective DNA-repair mechanisms, have been shown in both natural and pathogenic isolates of *E. coli* and *Salmonella enterica* [67, 68], and are very common among clinical isolates of *P. aeruginosa* [69] and *Neisseria meningitidis* [70, 71].

On a theoretical point, increased mutation rates increase the frequency of resistant phenotypes. Nevertheless, persistence of the mutator phenotype in a bacterial population is strongly affected by its adaptation to the environment. Under constant environmental conditions, mutators are unsuccessful because the negative effect of deleterious mutations on fitness outweighs that of the less frequent beneficial mutations. However, in new or fluctuating environments, such as in the different niches of an animal host, where bacteria face sequential bottlenecks and multiple mutations are needed for an adaptive character, mutators are more fit than nonmutators strains [72]. Moreover, the panmictic (as opposed to clonal) structure of certain microbial populations, (for instance, that of *N. meningitidis*) alleviates, on a population scale, the fitness burden of the mutator phenotype accounting for prevalence of mutator strains among hypervirulent lineages [70, 71].

Reacquisition of resistance through HGT is critical for at least two reasons. First, the high rate of HGT may seriously undermine reversal of resistance by supplying resistant genes from resistant to susceptible strains within the same population. Laboratory studies indicate that rate of plasmid transfer by conjugation may balance the rate at which plasmids are lost in *E. coli* populations [73]. If it were true in natural environments, the fate of a plasmid-borne resistance would be merely dependent on the fitness cost of the plasmid, which would determine the rate of persistence. Secondly, broad-host-range conjugative elements carrying antibiotic-resistant determinants might escape negative selection in their primitive host by rapid transfer into a secondary host that may be exposed to different selective pressure [74].

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6
Inhibitors of Cell-Wall Synthesis

Stefano Donadio and Margherita Sosio

6.1
Introduction

The formation of peptidoglycan, the inner layer of the cell wall, occurs through a complex pathway [1], which is a well-known and established target for antibacterials [2, 3]. Peptidoglycan consists of a 1,4-linked polysaccharide of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) units. A pentapeptide side chain, of general structure L-Ala-γ-D-Glu-X-D-Ala-D-Ala (where X can be L-Lys, *meso*-diaminopimelic acid, or another positively charged amino acid), is attached to the lactyl moiety of the MurNAc units. Peptidoglycan is cross-linked via transpeptidation between the terminal amino group of the charged amino acid at position 3 and the D-Ala residue at position 4 of a second strand, after removal of the fifth residue.

The earlier steps in the biosynthetic pathway occur in the cytoplasm and lead to the final cytoplasmatic precursor UDPMurNAc-pentapeptide (Figure 6.1) by the sequential action of the MurA-F enzymes and DdlB. This is then transferred by the membrane-bound translocase MraY onto undecaprenyl phosphate to give lipid intermediate I (Lipid I). Next, the glycosyltransferase MurG joins GlcNAc and the 4′-hydroxyl of MurNAc, to give lipid intermediate II (Lipid II). In some gram-positive bacteria, additional amino acids are added onto Lipid II from aminoacyl-tRNA donors, for example, five Gly residues in *Staphylococcus aureus* (Figure 6.1). The lipid-linked intermediate is transported onto the outer side of the cytoplasmic membrane, presumably by the action of a ''flippase,'' which has been only recently identified in *Escherichia coli* as the cell division protein FtsW [4]. Once on the cell surface, Lipid II is polymerized via transglycosylation and transpeptidation by the penicillin-binding proteins. Transglycosylation releases undecaprenyl-pyrophosphate, which is recycled via enzymatic dephosphorylation.

Peptidoglycan biosynthesis is targeted by several classes of antibiotics, although, at least judging from the microbial metabolites known so far, Nature seems to have preferred evolving inhibitors of the lipid-anchored biosynthetic steps (Figure 6.1). In fact, among the enzymes using free cytoplasmatic intermediates, only MurA and DdlB are targeted by fosfomycin and D-cycloserine, respectively [2].

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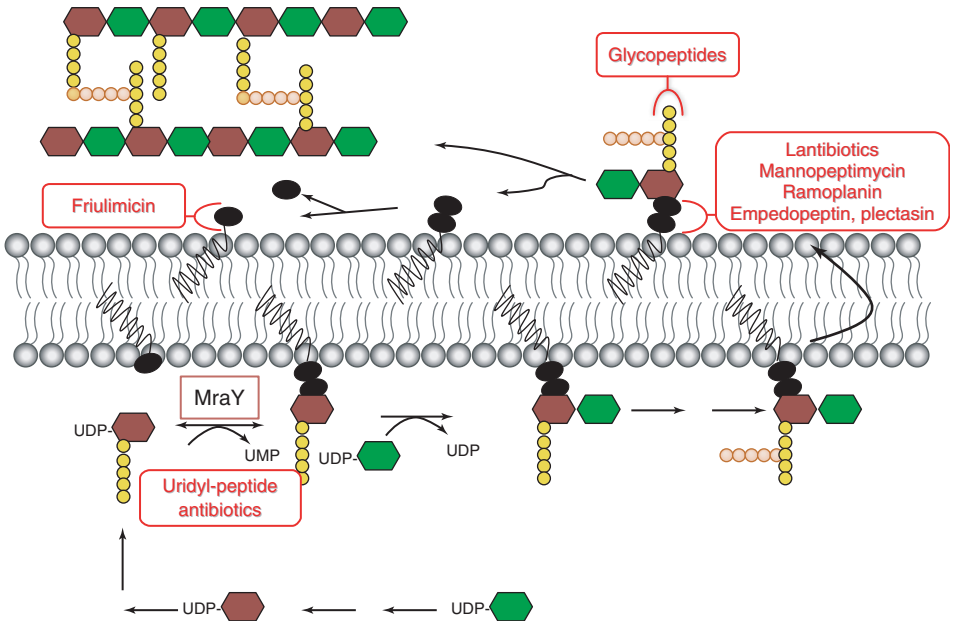


Figure 6.1 Schematic representation of cell-wall biosynthesis in *Staphylococcus aureus*, with details of membrane-associated steps and antibiotic target sites. Symbols: *N*-acetylglucosamine, green hexagons; *N*-acetylmuramic acid, brown hexagons; amino acid residues in pentapeptide chain, yellow balls; glycine residues in interpeptide bridge, orange balls; undecaprenyl unit, waved black lines; phosphate groups, black ovals. Antibiotics inhibiting key reactions are indicated. *Source*: Illustration adapted from Schneider and Sahl [3].

In contrast to later steps in the pathway (see subsequent text), these antibiotics are low-molecular weight, substrate-analog inhibitors. It should also be noted that high throughput screening (HTS) attempts at targeting the MurA-MurF steps have proved equally unproductive in providing compounds with decent antibacterial activity (reviewed in [2]).

Here, we focus our attention on recent developments of natural products targeting the translocase MraY, binding to Lipid II, and interfering with undecaprenyl pyrophospate recycling. The reader is referred to a number of recent, excellent reviews covering further aspects of peptidoglycan biosynthesis and its inhibitors [2, 3, 5]. We limit the description of chemical structures to selected examples; the reader is referred to Chapter 1 [6] for further details.

6.2 MraY Inhibitors

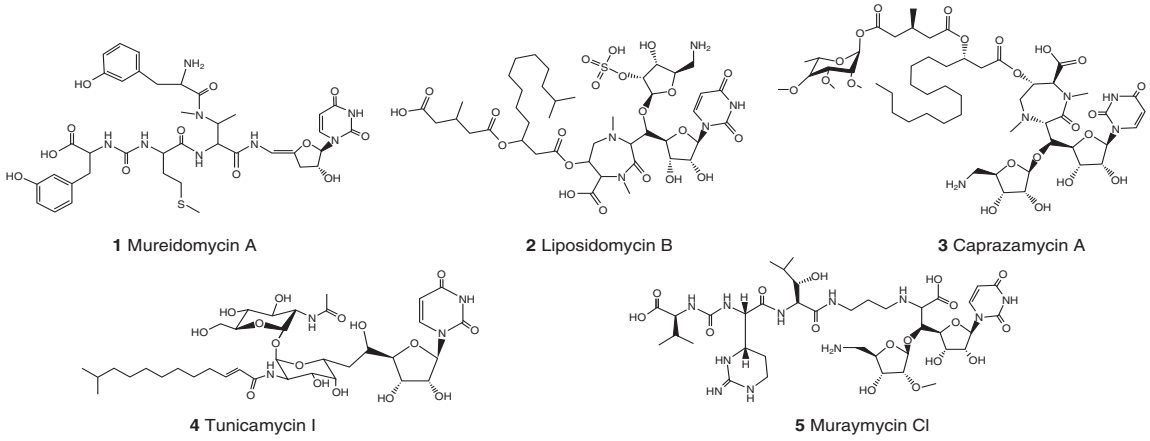
Antibiotics that target the MraY-catalyzed reaction are known as *uridyl peptide antibiotics* and include five families, with representatives for each family

(Figure 6.2): mureidomycins **1**, liposidomycins **2**, caprazamycins **3**, tunicamycins **4**, and muraymycins **5** [7].

Mureidomycins **1** (Figure 6.2) and related compounds (pacidamycins, napsamycins, and sansanmycins) share a common skeleton having an *N*-methyl 2,3-diaminobutyric acid (DABA) residue linked to a 3′-deoxyuridine nucleoside via a 4′,5′-enamide linkage. The presence of common structural moieties suggests a similar mode of action for this class of molecules. Despite good activity (MICs 4–16 μg ml^{-1}), pacidamycins did not protect mice against *Pseudomonas aeruginosa* infections [8]. Attempts at improving activity have so far met with limited success [9]. The mureidomycins also showed potent antibacterial activity against *Pseudomonas* (MIC 1.5–12.5 μg ml^{-1}) and weakly protected mice against *P. aeruginosa* infections, with ED_{50} values of 70–75 mg kg^{-1}, depending on congener [10]. Mureidomycin A inhibited MraY activity in *P. aeruginosa* preparations using a radiochemical assay (IC_{50}, 0.05 μg ml^{-1}), but not formation of lipid-linked *N*-acetylglucosamine for teichoic acid synthesis in *Bacillus subtilis* or mammalian glycoprotein biosynthesis [11]. Inhibition by mureidomycin A was found to be competitive versus both MraY substrates, UDPMurNAc pentapeptide and polyprenylphosphate [11]. Further structure–activity studies with several synthetic analogs have shown that the N-terminus of the antibiotic peptide chain interacts with the Mg^{2+} binding site in MraY [12].

The liposidomycins **2** (Figure 6.2), isolated from *Streptomyces griseosporeus* [13], contain uridine, aminoribose, diazepanone, and fatty acyl moieties and thus possess a structural resemblance to the substrates of MraY [7]. The caprazamyins **3** (Figure 6.2), also isolated from *Streptomyces* [14], differ from liposidomycins only in the absence of a sulfate group at the 2″-position of the aminoribose and in the presence of a permethylated L-rhamnose β-glycosidically linked to the 3-methylglutaryl moiety. Several other members of uridyl peptide antibiotics have been reported: the reader is referred to a number of recent papers covering biological and chemical aspects of these MraY inhibitors [3, 7, 15, 16].

The tunicamycins **4** (Figure 6.2) are structurally different from the other peptidyl nucleoside antibiotics, as they contain an additional GlcNAc moiety and a unique 11-carbon dialdose sugar (tunicamine), to which an *N*-linked fatty acid is attached. Tunicamycin is a reversible, competitive inhibitor for the sugar-nucleotide substrate of MraY but has no effect on the rate of polyprenolphosphate binding [17]. Modeling studies have indicated that, as a structural analog of the UDP-D-HexNAc substrate, tunicamycin functions by mimicking the uracil diphosphate (UDP)-sugar, with the *N*-glycosidically-linked uracil, reversibly coordinating the divalent metal cofactor in the translocase active site [18]. This is not the only distinctive feature of tunicamycin, which also inhibits eukaryotic UDP-*N*-acetylglucosamine:dolichol phosphate GlcNAc-1-P transferase, the enzyme catalyzing the first step in protein glycosylation, and *N*-palmitoylation of acyl proteins, showing high mammalian toxicity [7]. While tunicamycin has become a useful tool for studying protein glycosylation in eukaryotes, its promiscuous activity has adversely affected its use as an antibiotic. Recent studies indicate that low levels of tunicamycin can inhibit the translocase TarO involved in wall teichoic acid biosynthesis in *S. aureus* without



1 Mureidomycin A

2 Liposidomycin B

3 Caprazamycin A

4 Tunicamycin I

5 Muraymycin CI

Figure 6.2 Representative inhibitors of MraY.

affecting peptidoglycan synthesis. A synthetic lethal combination of tunicamycin with a β-lactam has been observed in methicillin-resistant *Staphylococcus aureus* (MRSA) [19].

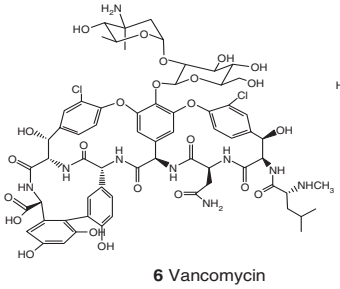
The muraymycins **5** (Figure 6.2), also produced by *Streptomyces*, were identified by cell-wall-specific bioassays [20]. Members of this family, including recently synthesized analogs, show broad-spectrum antimicrobial activity against a variety of clinical isolates (MIC 2 to $> 64\,\mu g\,ml^{-1}$), with muraymycin A1 also protecting mice from an *S. aureus* infection with an ED_{50} of $1.1\,mg\,kg^{-1}$ [20]. The core structure of muraymycins contains a glycosylated uronic acid derivative joined by an aminopropane group to a hexahydro-2-imino-4-pyrimidylglycyl-containing dipeptide that is further extended by a urea-valine moiety. Structure–activity relationship studies have been carried out using synthetically produced analogs, leading to a model in which the inner moiety of the urea-dipeptide motif interacts with the carbohydrate recognition domain of MraY [21]. The fatty acid substituent and the presence or the absence of the amino sugar also play important roles in biological activity [22].

6.3 Lipid II Targeting Compounds

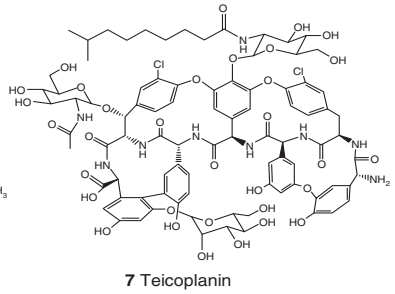
The key biosynthetic intermediate Lipid II is directly complexed by several classes of antibiotics, with resulting inhibition of cell-wall biosynthesis. For many compounds, the pharmacophore involved in Lipid II binding actually represents a small portion within a relatively large, complex molecule. For some of these antibiotics, binding to Lipid II is followed by events that enhance antibacterial activity.

6.3.1 Glycopeptides

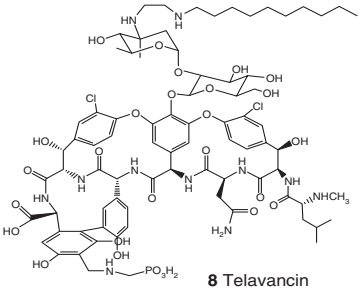
Vancomycin **6**, teicoplanin **7**, and telavancin **8** (Figure 6.3) are the clinically approved drugs within the glycopeptide antibiotics, molecules of last resort for treating infections caused by multidrug-resistant (MDR) gram-positive pathogens. Vancomycin, discovered in 1955 at Eli Lilly from *Amycolatopsis orientalis*, was approved in 1958 for clinical use in the United States; teicoplanin was isolated at Lepetit from *Actinoplanes teichomyceticus* and approved in 1988 for clinical use in Europe and subsequently in Japan; and telavancin, a semisynthetic derivative of vancomycin, was approved in 2009 to treat complicated skin infections that are suspected or confirmed to be caused by MRSA. Other glycopeptides have entered clinical development [23]: oritavancin **9** (Figure 6.3), a semisynthetic derivative of a vancomycin analog, whose development for systemic use appears to have been discontinued because of unsatisfactory results from a phase 3 study; and dalbavancin **10** (Figure 6.3), a semisynthetic derivative of the teicoplanin-related glycopeptide A40926, whose marketing authorization will require a further phase 3 clinical study.



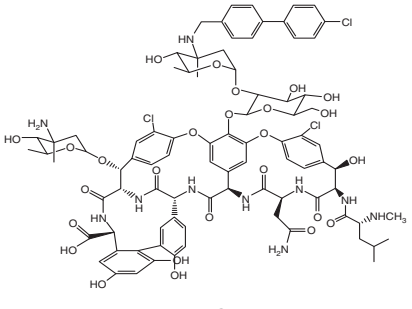
6 Vancomycin



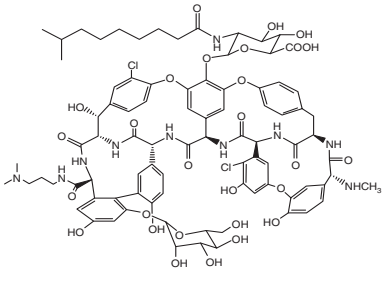
7 Teicoplanin



8 Telavancin



9 Oritavancin



10 Dalbavancin

Figure 6.3 Representative glycopeptides.

Despite different decorations on the cross-linked heptapeptide scaffold introduced by the producing strains (most notably, sugars or a long acyl chain linked to the aminosugar) or by semisynthesis (cf. structures **6–10** in Figure 6.3), all glycopeptides share the same pharmacophore, involved in forming a tight complex with the D-Ala-D-Ala terminus of the peptide side chain of Lipid II through five hydrogen bonds [6]. In this way, glycopeptide sequester Lipid II on the external face of the bacterial membrane from enzymes involved in later steps in peptidoglycan biosynthesis. This causes failure to form cross-links, lowering the rigidity of the cell wall and rendering the cell susceptible to osmotic lysis.

While binding to the D-Ala-D-Ala termini of Lipid II is the primary mechanism by which glycopeptides exert their action, other factors appear to contribute to their antibacterial activity. Vancomycin **6** and related compounds are able to form dimers *in vitro*, and this has led to the suggestion that dimers, by acting as a single entity in recruiting two molecules of the target, possess an enhanced affinity for Lipid II. However, while a correlation between dimerization and antibacterial activity holds for structurally related compounds, it is weaker when comparing different glycopeptides [24, 25]. Antibiotics of the teicoplanin family, instead, appear to possess a second, weaker binding site, represented by their anchoring onto the bacterial phospholipid bilayer through their acyl chains [26]. Additional binding sites have been exploited in some of the second-generation glycopeptides, which possess activity against vancomycin-resistant enterococci (VRE). This is mainly achieved by the addition of hydrophobic moieties to the glycopeptide structure, which compensate for reduced binding to D-Ala-D-Lac-ending intermediates. For example, telavancin **8** has been shown to also act through the disruption of bacterial membrane integrity, a mechanism usually not seen with other glycopeptides [27]. A similar mechanism may operate with oritavancin **9**, with the chlorobiphenylmethyl substituent on the disaccharide allowing cell membrane anchoring and stabilization of the interaction with Lipid II. Oritavancin's activity versus VRE strains may also benefit from a secondary binding site to the pentaglycyl bridging segment [25]. Dalbavancin **10**, which carries a basic amide at the C-terminus of the heptapeptide, exhibits improved potency against staphylococci in comparison to teicoplanin **7**. The long lipophilic side chain of dalbavancin and its increased positive charge help anchor the compound to the membrane, leading to effective inhibition of PBP2 in *S. aureus* [28]. Furthermore, dalbavancin's favorable pharmacokinetic suggests a once-weekly dosing in humans.

6.3.2 Lantibiotics

The lanthipeptides are ribosomally synthesized, posttranslationally modified peptides containing the characteristic thioether cross-links. Among the lanthipeptides are lantibiotics, which possess antibacterial activity [29]. Actually, most of the lanthipeptides discovered are lantibiotics, but this may also be due to a bias in the bioassays used to detect them. Lantibiotics vary greatly in their antibacterial activity,

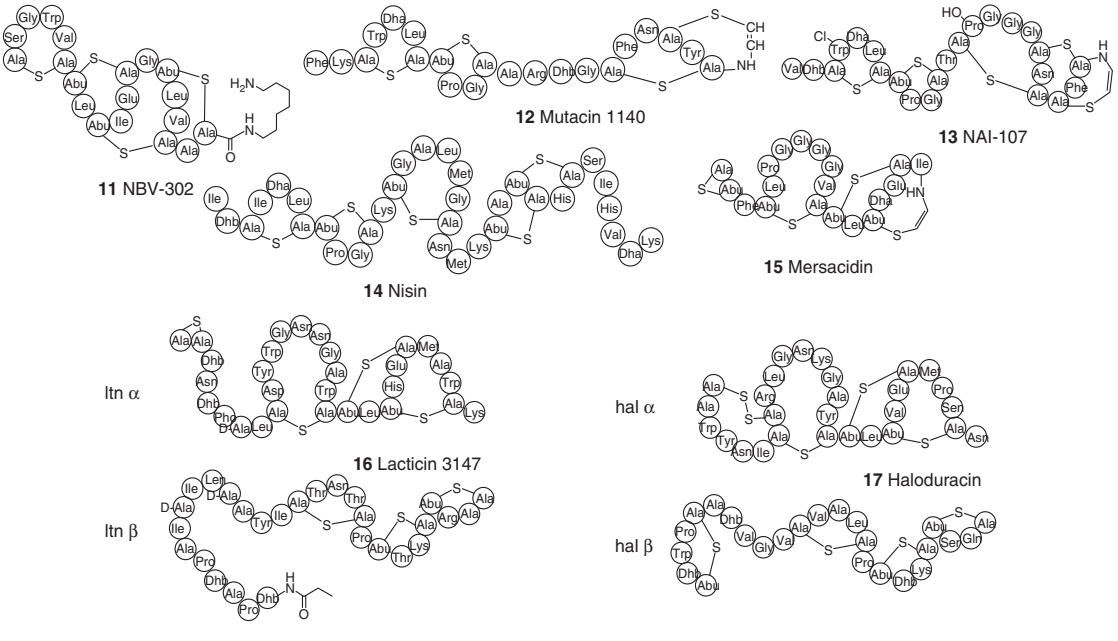


Figure 6.4 Representative lantibiotics.

but some compounds can exhibit potent activity against clinically relevant gram-positive bacteria, including drug-resistant strains of *Staphylococcus*, *Streptococcus*, *Enterococcus*, and *Clostridium*. Notwithstanding these promising features, few lantibiotics have advanced into development. Notable exceptions are represented by NBV-302 **11** (Figure 6.4), a derivative of actagardine in phase 1 for the treatment of *Clostridium difficile* infections [30]; mutacin 1140 **12** (Figure 6.4), under late preclinical development [31]; and NAI-107 **13** (Figure 6.4), efficacious in several models of experimental infection caused by MDR gram-positive pathogens [32], also under late preclinical development.

While biogenetic and structural considerations group lanthipeptides into four different classes, relevant antibacterial activities are found only among members of classes I and II [29]. Class I lanthipeptides, exemplified by nisin **14** (Figure 6.4), are generally screw shaped, elongated, flexible, and amphiphatic with an overall positive net charge and pore-forming activities (with exceptions, see subsequent text). Class II lanthipeptides, for example, mersacidin **15** (Figure 6.4), are generally rigid globular molecules that carry either no net charge or no net negative charge. Unique within the class II molecules are two-component lantibiotics, such as lacticin 3147 **16** (Figure 6.4) and haloduracin **17** (Figure 6.4), in which two peptides, each encoded by its structural gene and processed by the cognate enzyme, act synergistically to form the mature antibiotic [29].

The mechanisms by which antibacterial lanthipeptides exert biological activity have been studied extensively in only a few instances, but they are all believed to inhibit cell-wall biosynthesis by binding to Lipid II and, in some instances, disrupt membrane integrity through pore formation.

The formation of a nisin:Lipid II complex leads to inhibition of transglycosylation, with a profound impact on the entire cell-wall synthesis machinery, resulting in delocalization of this precursor from the septum, aberrant septum formation, and most likely the disorganization of multimeric protein complexes [33]. The interaction primarily involves the formation of five hydrogen bonds between the pyrophosphate moiety of Lipid II and the A and B rings of nisin **14**, which are conserved in several class I lanthipeptides, including NAI-107 and mutacin 1140 (Figure 6.5a). Once bound to Lipid II, a transmembrane orientation of nisin occurs involving insertion of its C-terminal part and a flexible hinge region, leading to the formation of stable pores in a complex consisting of eight nisin and four lipid II molecules [33]. This causes dissipation of the membrane potential, rapid efflux of small metabolites, and rapid cell lysis. Many class I lantibiotics contain the *N*-terminal rings involved in pyrophosphate caging but lack the C-terminal tail and do not form pores or form pores in a strain-specific manner, dependent on the length of membrane phospholipids [29]. For example, binding of bovicin HC5 to Lipid II inhibits the pore-forming activity of nisin, presumably by sequestering most Lipid II binding sites [34]. NAI-107 **13**, despite its potent antibacterial activity, binds to Lipid II but does not form pores (D. Münch *et al.*, unpublished results).

The class II lanthipeptides mersacidin **15** also binds to Lipid II and inhibits transglycosylation in a Ca^{2+}-dependent manner [37]: binding involves a conformational change that is probably dependent on movement around a hinge region located at

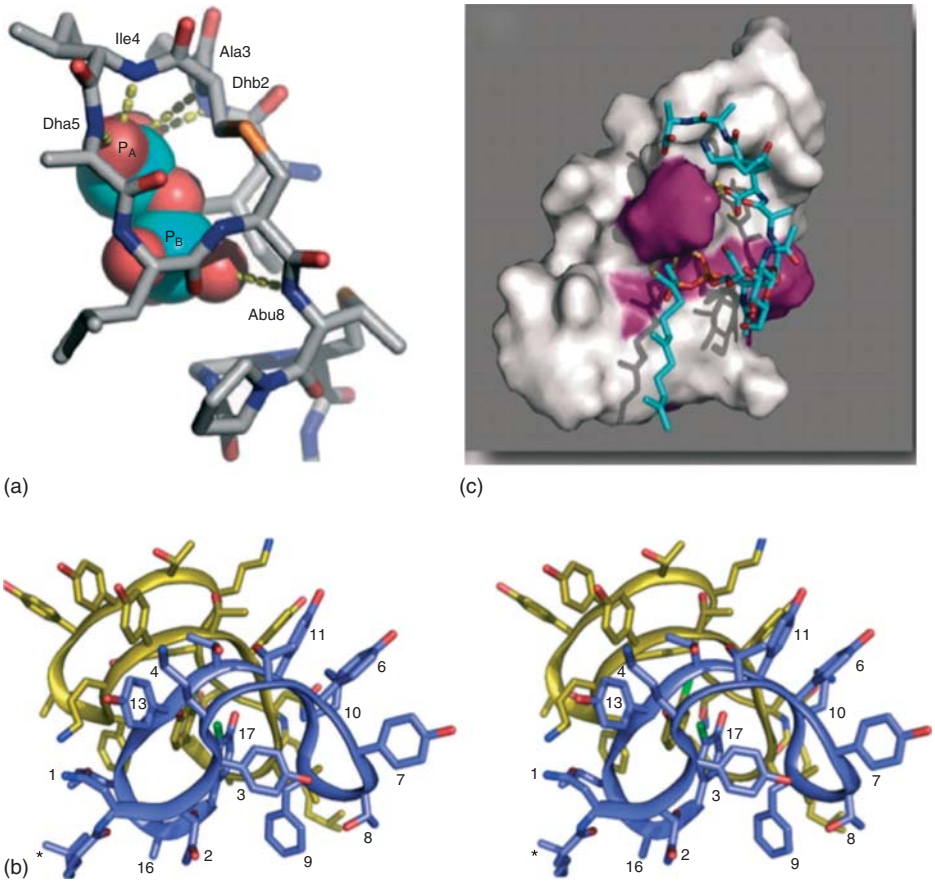


Figure 6.5 (a) Detailed view of the nisin backbone with the pyrophosphate-binding pocket. In this model, the intermolecular hydrogen bonds are indicated by yellow dashed lines and the corresponding nisin residues are labeled with the pyrophosphate group represented as spheres. The sulfur atoms in the lanthionine rings are orange. *Source*: The illustration is from Hsu *et al*. [33]. (b) Stereoview of ramoplanin dimer (one monomer labeled blue and the other orange) with selected side chains of one monomer labeled. Asterisk indicates the fatty acid moiety. *Source*: The illustration is from Hamburger *et al*. [35]. (c) NMR-based model of the plectasin/Lipid II-complex. Plectasin residues believed to be involved in binding are highlighted in magenta. *Source*: The illustration is from Schneider *et al*. [36].

residues 12 and 13. Mersacidin and other class II lantibiotics lack the ability to form pores. For the two-component lantibiotics, the individual peptides display little or no activity alone, but afford potent and synergistic activity in combination [29]. The α-peptide of haloduracin **17** shares the mersacidin-binding motif and interacts with Lipid II, where it benefits from the presence of the β peptide, which facilitates pore-formation. The complex has been shown to consist of Lipid II:Halα:Halβ in a 1 : 2 : 2 stoichiometry [38]. A similar mechanism is believed to operate in lacticin 3147 **16**.

6.3.3
Ramoplanin and Enduracidin

Ramoplanin **18** and enduracidin **19** (Figure 6.6), produced by *Actinoplanes* sp. and *Streptomyces fungicidus*, respectively, consist of 49-membered macrolactones formed from a 17-aa peptide, which includes several nonproteinogenic amino acids. They are further decorated with an acyl chain and, in the case of ramoplanin, with a di-mannose moiety. While enduracidin is sold commercially as a feed additive for broiler chickens, ramoplanin has been in development for the treatment of *C. difficile* infections.

Ramoplanin inhibits the transglycosidases by binding and sequestering Lipid II at the interface between the extracellular environment and the bacterial membrane [35]. Binding experiments with short-chain variants have shown that ramoplanin binds any variant of Lipid II or Lipid I provided that it contains a pyrophosphate, even as simple as farnesyl pyrophosphate [39]. The crystal structure of ramoplanin A2 (Figure 6.5b) has unveiled the occurrence of a highly amphipathic dimer, suggesting a mechanism by which ramoplanin recognizes its ligand and how it interacts with bacterial target membranes: residues 3 through 10 play a crucial role in the interaction with the MurNAc and pyrophosphate moieties of Lipid II [35]. Alanine scanning (i.e., synthesis of ramoplanin variants with alanine at different positions) identified Orn-10, Hpg-3, Hpg-7, and Orn-4 (listed in order of decreasing importance) as the residues critical for binding [40]. Another group important for activity is the lipid tail: while analogs completely devoid of the acyl chain maintain an affinity for Lipid II similar to that of ramoplanin, their antibacterial activities decrease at least 10-fold [40], suggesting that the lipid tail plays an important role in enhancing ramoplanin potency by targeting it to the bacterial membrane. The lipid tail appears also to be responsible for ramoplanin's hemolytic activity: its replacement with a variety of carboxylic acids yielded analogs with improved tolerability profile but antibacterial activity equivalent to that of the parent compound [41].

6.3.4
Other Compounds

Empedopeptin **20** (Figure 6.6), an amphoteric cyclic lipodepsipeptide produced by the Bacteroidetes *Empedobacter haloabium*, shows potent activity against a broad range of aerobic and anaerobic gram-positive bacteria. While empedopeptin shows affinity for additional bactoprenol-containing precursors that occur outside the cell, Lipid II is believed to be its primary and main interaction site. However, if bacterial cells are exposed to sufficiently high concentrations, empedopeptin can recognize the other structures, forming remarkably stable 2 : 1 antibiotic to lipid precursor complexes [42]. The presence of Ca^{2+} ions is required for full inhibitory activity of empedopeptin in *in vitro* peptidoglycan synthesis assays.

Mannopeptimycin **21** (Figure 6.6), isolated from *Streptomyces hygroscopicus*, is produced as a complex of five main congeners characterized by a cyclic hexapeptide

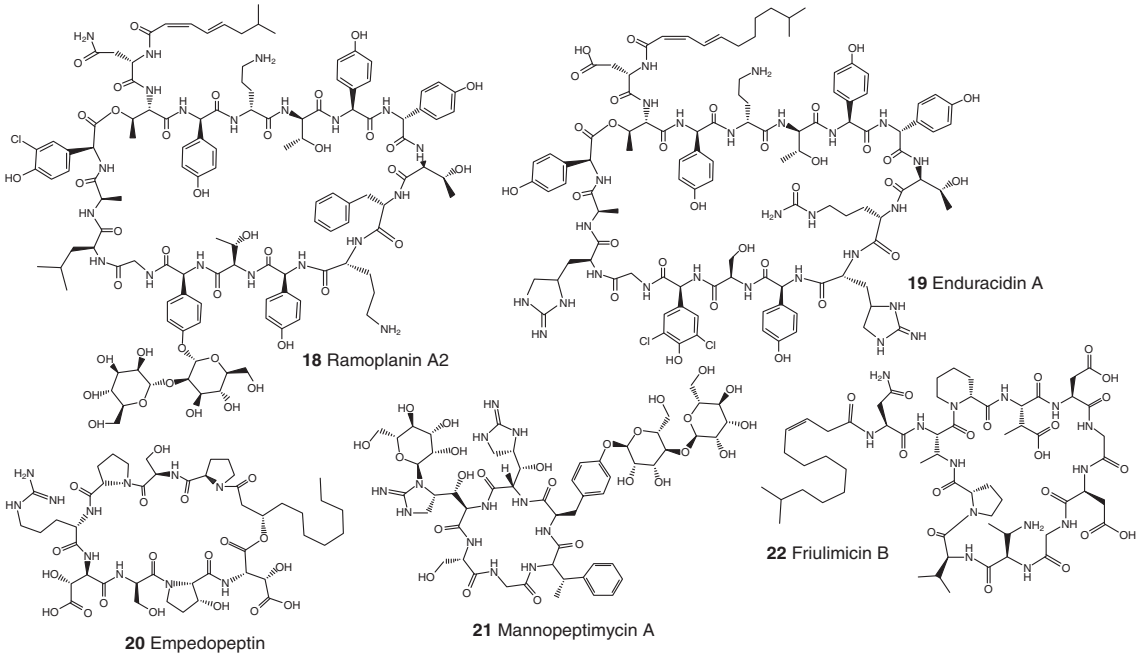


Figure 6.6 Other cell wall inhibitors described in the text.

core, glycosylated mannose units, with some congeners carrying an isovaleryl group attached to the terminal mannose. A decade ago, mannopeptimycin was reevaluated for its efficacy against clinically important gram-positive pathogens, including MRSA, penicillin-resistant *Streptococcus pneumoniae*, and VRE. It acts by blocking the transglycosylation step necessary for incorporation of Lipid II into nascent peptidoglycan but differently from other Lipid II-binders. [^{3}H]-mannopeptimycin binds to both isolated and membrane-bound Lipid II and it also interacts with lipoteichoic acid, likely interfering with improper transfer of functional groups to teichoic acid and thus blocking teichoic acid biosynthesis [43].

Plectasin, originally isolated from the saprophytic ascomycete *Pseudoplectania nigrella* and later produced in recombinant form by *Aspergillus oryzae*, is a 95-aa peptide, consisting of a signal sequence (residues 1–23), a prosegment (residues 24–55), and a 40-residue C-terminal domain. Its primary, secondary, and tertiary structures closely resemble those of invertebrate defensins with which it shares the disulfide bridges and a cationic amphiphilic character [44]. While plectasin is active especially against *S. pneumoniae*, the improved derivative NZ2114 covers also staphylococci [45]. In *in vivo* studies, plectasin showed low toxicity in mice, and was efficacious in experimental peritonitis and pneumonia caused by *S. pneumoniae*. Contrary to defensins that act by binding and disrupting bacterial cytoplasmic membrane integrity, several elegant biochemical and genetic experiments proved that plectasin interferes with cell-wall biosynthesis [36]. In particular, plectasin-treated *Staphylococcus simulans* cells accumulate UDP-MurNAc-pentapeptide, while a 1 : 1 stoichiometric complex between Lipid II and plectasin is formed *in vitro*. Nuclear magnetic resonance spectroscopy and computational modeling led to the identification of key plectasin residues involved in complex formation, supporting a model in which plectasin gains affinity and specificity through binding to the solvent-exposed part of Lipid II, whereas the hydrophobic part of plectasin is located at the membrane interface (Figure 6.5c). Remarkably, plectasin shares functional features with nisin, as both compounds recognize the pyrophosphate moiety of Lipid II, but their action diverge after this first docking event.

6.4 Bactoprenol Phosphate

The amphomycin family of lipopeptide antibiotics includes several members, among which friulimicin **22** (Figure 6.6) is probably the best characterized. It is produced by *Actinoplanes friuliensis* and exhibits potent antibacterial activity against a number of gram-positive pathogens, including MRSA, penicillin-resistant pneumococci, VRE, and *C. difficile* [46]. Frilumicin has been in phase 1 clinical trials, although further development at the moment has been halted [23]. Assays using membrane preparations of *Micrococcus luteus* with defined amounts of the soluble precursors UDP-MurNAc-pentapeptide and UDP-GlcNAc established that friulimicin blocks lipid II formation [47]. Complete inhibition was observed upon addition of at least equimolar concentrations of friulimicin and the C_{55}-P carrier. All data

strongly support the hypothesis that friulimicin specifically forms a Ca^{2+}-dependent complex with the monophosphorylated bactoprenol carrier without affecting membrane integrity. A model has been proposed in which friulimicin, after coordination of the Ca^{2+} ions, forms a dimer that creates a tunnel-like structure with hydrophobic and hydrophilic areas, which is able to accommodate bactoprenol-phosphate. Because of the calcium ions, the openings of the tunnel are positively charged and may further strengthen the negatively charged phosphate group in the target.

6.5 Conclusions

In this overview, we have focused our attention on the antibiotics that inhibit peptidoglycan biosynthesis by acting at or close to the bacterial membrane, in both the inner and the outer side of it. Energy-intensive reactions occur in the cytoplasm and substrates are loaded on the carrier and flipped on the outer side of the membrane, where they are polymerized on the existing peptidoglycan, while the unloaded carrier is flipped back to the inner side of the membrane. Around this ''membrane cycle,'' Nature has devised several compounds that can inhibit this critical process at different steps.

Perhaps, the most remarkable finding is that six unrelated classes of microbial products (glycopeptides, lantibiotics, ramoplanin, empedopeptin, mannopeptimycin, and plectasin) exert their inhibitory activity by binding to relatively small portions of Lipid II. In many cases, this first docking event is followed by dimerization and/or membrane anchoring events that increase the affinity of the inhibitor. It is also remarkable that Lipid II-binding antibiotics have relatively high molecular weight, in many cases larger than that of their target. Apparently, targeting a small biosynthetic intermediate often requires a larger antibiotic than targeting a macromolecule such as an enzyme.

One recurring question in the minds of those searching for novel antibiotics is whether all the low-hanging fruit have already been picked and those left to be discovered might be at an unreachable height. Several of the compounds described in this chapter were actually reported in the past decade or so, suggesting that focused efforts in natural products can still provide pleasant surprises. At the same time, it appears that not all targets have been created equal [2], at least from Nature's point of view. For reasons we do not yet understand, there is apparently a higher probability of finding a new MraY or Lipid II inhibitor than a natural product acting on, say, MurC or MurF. However, we would be happy to be contradicted on this topic by future findings.

Acknowledgments

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7
Inhibitors of Bacterial Cell Partitioning

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7.1
Introduction

The ever-increasing emergence of various drug-resistant bacterial strains is posing serious health concerns. Surfacing of several new pathogenic strains in recent years has further aggravated this problem. The extensive and reckless usage of antibiotics has led to the emergence of drug-resistant bacterial strains. As a consequence, the currently available drugs have poor clinical outcomes in treating bacterial diseases. The situation has become alarming and there is a desperate need for antibiotics with novel cellular targets. Bacterial cell division machinery is one such target, which, despite being indispensable for the bacterial cell survival, has remained relatively unexploited for developing antibiotics. Nevertheless, the therapeutic potential of targeting bacterial cell division components is now being realized and explored. Recent studies have suggested that the perturbation of the assembly dynamics of FtsZ, a central component of the bacterial cell division machinery, leads to inhibition of bacterial cell proliferation [1–3]. Various natural and synthetic compounds that prevent bacterial cell division by targeting FtsZ have been identified [1–3].

Most of the components of bacterial cell division machinery, including FtsZ, are conserved across the bacterial kingdom and are essential for the process of cytokinesis [4]. Inhibitors targeting the cell division process certainly provide new avenues for developing new and effective antibacterials [5]. In this chapter, we have first provided a brief description of the process of bacterial cell division with an emphasis on the proteins involved and their functions. Subsequently, various known inhibitors of bacterial cell partitioning are discussed. The potential of targeting various accessory proteins of bacterial cell division for therapeutic purposes along with an overall perspective on the current status and future scope of the field are provided.

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7.2 Bacterial Cell Division

Bacteria most commonly divide by the process of binary fission, wherein a mother cell splits into two identical daughter cells. The process broadly involves chromosome duplication, chromosome segregation, and cell partitioning (cell division). Despite having independent control systems, these events are well coordinated in a bacterial cell cycle [6]. Bacterial cell division is a complex, yet highly regulated, process that requires the participation of various proteins, of which FtsZ is a central component. It forms a ring like structure called *Z-ring* at the center of the cell [7]. The Z-ring is the foundation for the future division septum; a group of proteins is recruited in succession at the Z-ring, leading to the formation of a complex divisome that orchestrates bacterial cytokinesis [8–10].

7.2.1 Filamentous Temperature-Sensitive Z (FtsZ)

The FtsZ gene was discovered in the late 1970s during the characterization of some *Escherichia coli* mutants. It was earlier observed that a few mutations in *E. coli* caused filamentation in the cells at restrictive temperatures, and the gene where they mapped was named as *ftsA* [11–13]. As the mutation in the gene led to filamentous morphology of cells and the phenotype was sensitive to temperature, the gene got the prefix ''fts'' (filamentous temperature sensitive) [12]. Later on, one more mutation was identified, which was causing the filamentous morphology in cells but it did not map in the *ftsA* gene. The gene having this mutation was named as *ftsZ* [14]. The results of complementation assay suggested that this gene is located between *ftsA* and *envA* genes [14]. Subsequently, it was established that *ftsZ* is essential for bacterial division [15] and is involved in the earliest step of the division process [16, 17].

7.2.2 Structure and Assembly Properties of FtsZ

FtsZ belongs to the family of GTPases with a guanosine-5′-triphosphate (GTP)-binding motif (GGGTGTG) similar to that of a eukaryotic cytoskeletal element, tubulin (GGGTGST) [18–20]. It binds and hydrolyzes GTP and undergoes self-assembly to form higher order structures. The polymerization of FtsZ occurs in a GTP-dependent manner and requires monomer concentration above a threshold level known as *critical concentration* [21]. The polymerization leads to the formation of straight GTP-bound filaments. GTP hydrolysis leads to filament curvature due to replacement of GTP by GDP and results in their depolymerization [22–24]. Analysis of the crystal structure of FtsZ revealed that it consists of two domains; N and C joined by a central (H7) helix [24, 25]. The N-domain consists of the GTP-binding motif and the C-domain has a loop structure, termed as *synergy (T7) loop*, which is responsible for GTP hydrolysis. The interaction of the T7 loop of an FtsZ

monomer with the nucleotide-binding pocket of another FtsZ monomer creates the GTPase active site [24, 25]. The longitudinal association of FtsZ monomers leads to the formation of long FtsZ protofilaments. These filaments associate laterally to form bundles, which fabricate the Z-ring (Figure 7.1). The energy of longitudinal and lateral interactions has been estimated to be 7–23 k_BT per bond and 0.1–0.3 k_BT per monomer, respectively, under *in vitro* conditions [23].

7.2.3 Z-Ring: A Dynamic Structure That Drives Bacterial Cell Division

FtsZ forms the fundamental cell division structure ''Z-ring'' at the center of the cell [7]. In a newly divided cell, FtsZ remains in the form of monomers in cytoplasm. As the nucleoids start segregating after duplication, monomers of FtsZ start migrating toward the cell center, wherein they polymerize with the aid of regulatory proteins to form Z-ring (Figure 7.1; [8, 10]). At first, Bi and Lutkenhaus identified a ring like structure composed of FtsZ inside the cells using immunoelectronmicroscopy [7] and, subsequently, the localization of FtsZ at the midcell was confirmed using immunofluorescence and green fluorescent protein (GFP)-tagged FtsZ [27, 28]. A high-resolution *in vivo* image of the Z-ring is still elusive but recently photoactivated localization microscopy (PALM) disclosed that the Z-ring is composed of overlapping bundles of FtsZ and the thickness of the Z-ring was measured to be ~110 nm [29].

The Z-ring is a highly dynamic structure that continuously exchanges its GDP-bound FtsZ monomers, formed as a result of GTP hydrolysis with GTP-bound FtsZ monomers from the cytoplasmic pool. Using fluorescent recovery after photobleaching (FRAP) experiments, the Z-ring was shown to remodel itself continuously in the cells with a halftime of ~30 s [30]; however, more recent experiments have indicated the turnover rate to be 9 s [31]. Studies have been performed to decipher the correlation between FtsZ polymerization and its GTPase activity [22, 30, 31]. *In vivo* experiments with *E. coli* cells expressing FtsZ mutant (*ftsZ84*) with 10-fold lesser GTPase activity revealed that it could form Z-ring in the cells; however the Z-ring had a drastically (~ninefold) reduced turnover rate [30]. This suggests that the hydrolysis of GTP is not required for FtsZ assembly but is crucial for maintaining Z-ring dynamicity.

The Z-ring not only forms the framework for cytokinetic ring but also plays an important role in cell constriction (Figure 7.1). With the aid of *in vitro* reconstitution experiments and simulation studies, it has become possible to study the mechanism of force generation by Z-ring constriction [32, 33]. It has been found that FtsZ could form rings inside the liposomes in the presence of GTP and these rings could even cause a constriction in the liposomes [34]. The constriction force generated by the Z-ring is attributed to the lateral interactions present between the protofilaments and filament curvature. *In silico* simulation studies have shown that the Z-ring undergoes a condensation wherein a decrease in the Z-ring diameter is coupled with an increase in its thickness, providing enough force for mediating cell constriction [35]. No net change in the number of FtsZ

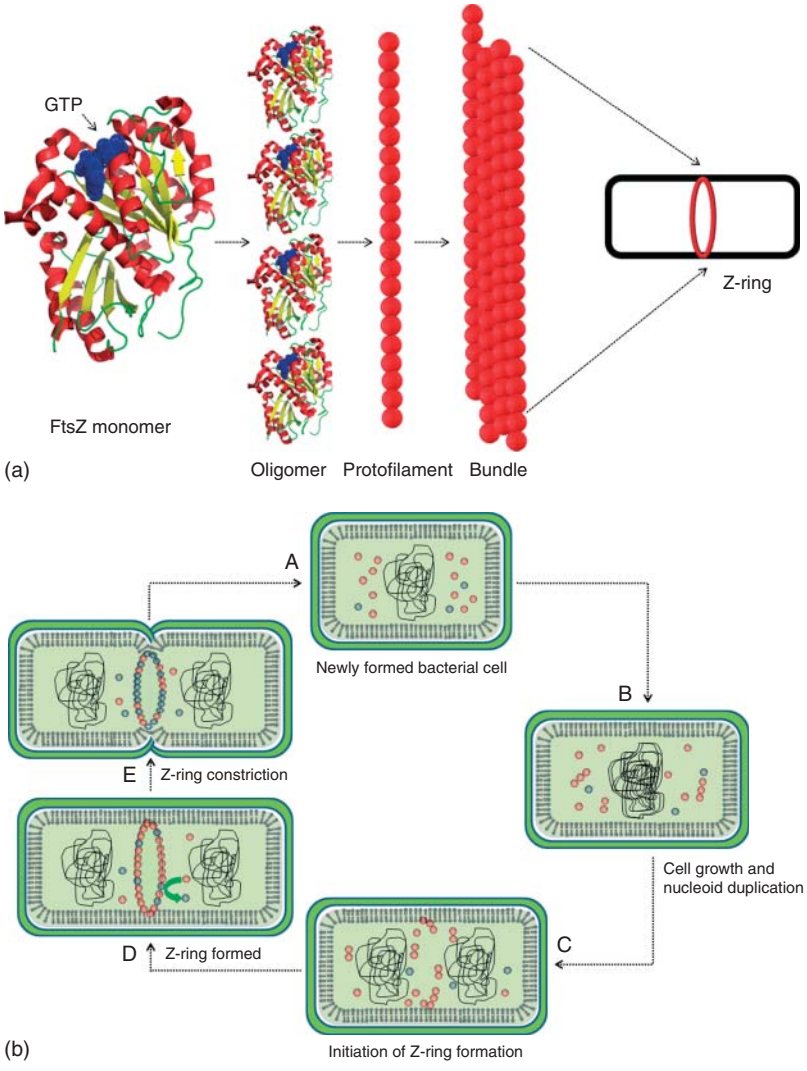


Figure 7.1 (a) Z-ring assembly – FtsZ monomers polymerize into filaments in the presence of GTP to form the Z-ring. The monomer structure has been taken from the protein data bank (PDB ID – 2VAM) [24] and then drawn in PyMol [26]. (b) FtsZ assembly dynamics in bacterial cytokinesis. (A) A newly divided bacteria having FtsZ monomers in the cytoplasm. (B) The bacterial cell grows and the duplication of nucleoid occurs. (C) The duplicated nucleoids start separating, creating a space at the center of the cells wherein FtsZ monomers migrate and initiate the formation of the Z-ring. (D) A complete Z-ring is formed at the center of the cell. There occurs a continuous exchange of GDP-FtsZ in the ring with GTP-FtsZ from the cytoplasm. (E) Finally, the constriction of the Z-ring starts and septum synthesis occurs, which leads to cytokinesis, resulting in the formation of two daughter cells. Red and blue circles show GTP- and GDP-bound FtsZ monomers, respectively.

monomers occurs during this transition and the increase in the Z-ring density solely occurs because of its contraction. The morphological changes in the ring during the condensation are due to the continuous turnover of monomers as a result of the GTP hydrolysis [23].

7.2.4 Proteins Regulating FtsZ Assembly

The formation and functioning of the Z-ring is not as simple as it appears. Several proteins have been suggested to assist in the assembly and proper functioning of the Z-ring in bacteria. In the initial stages of its formation, there are proteins that govern the site of Z-ring formation and ensure that it is positioned correctly at the center of the cells. This spatial localization of the Z-ring at the mid-cell position is dependent on the nucleoid occlusion and Min systems [36]. Nucleoid occlusion proteins comprising SlmA in *E.coli* and Noc in *Bacillus subtilis* prevent the formation of the Z-ring over the nucleoids [37–39]. Thus, the nucleation of the Z-ring occurs once the duplicated nucleoid starts separating, creating a space for the influx of FtsZ monomers. Alternatively, Min proteins prevent the formation of Z-ring at the cellular poles. The Min protein complex consists of MinCDE in *E.coli* and MinCDJ with DivIVA in *B. subtilis* [40–42]. Inside the cell, the Min proteins are distributed as a gradient, their concentration being highest at the poles and lowest at the mid cell [43, 44]. Because MinC in association with MinD inhibits the assembly of FtsZ, the formation of the ring is therefore restricted to the mid-cell position [41].

In addition, a set of proteins helps in the establishment of the Z-ring. FtsZ lacks a membrane-binding domain; therefore, it requires anchors to attach itself to the cell membrane. FtsA and ZipA accomplish this task and also stabilize the Z-ring [45, 46]. FtsA, an ATPase, is a membrane-anchoring protein that tethers the Z-ring to the membrane. It also promotes the assembly of FtsZ, thereby stabilizing the Z-ring. FtsA is implicated in the recruitment of various other downstream proteins involved in the formation of the divisome [47–49]. In *E. coli*, ZipA, in association with FtsA, tethers the Z-ring to the membrane and further stabilizes it [45, 50–52].

Several other proteins are involved in the regulation of FtsZ assembly dynamics and their mechanism of regulation is well studied. These accessory proteins are recruited in various stages of the division process and regulate the Z-ring formation and dynamics depending on their mechanism of action. These proteins mainly comprise SepF, ZapA, ZapB, EzrA, ClpX, UgtP, MciZ, and SulA [10]. SepF, ZapA, and ZapB promote the polymerization of FtsZ and thereby stabilize the Z-ring [53–55]. In addition, recent studies have indicated the existence of two more positive regulators, ZapC and ZapD in *E. coli* [56, 57]. ZapC colocalizes with the Z-ring at the mid cell and promotes the lateral association of FtsZ [56]. ZapD also directly interacts with FtsZ and promotes its assembly and bundling [57]. In contrast, ClpX inhibits the formation of the Z-ring in bacterial cells and maintains the concentration of FtsZ monomers in the cytoplasm [58, 59]. EzrA (extra Z-ring A),

a negative regulator of FtsZ assembly, inhibits the polymerization of FtsZ, thus preventing the formation of extra Z-rings at the cellular locations other than the mid cell [60, 61]. In addition, UgtP, MciZ, and SulA are cell-responsive regulators, which are expressed according to the physiological status of the cell [10]. UgtP, a terminal sugar transferase, in *B. subtilis* is expressed more in the cell under nutrient-rich conditions and its main function is to inhibit the assembly of FtsZ in order to delay the division process till the cell attains a proper size [62]. SulA, an SOS response protein, is expressed in the cell during DNA damage and perturbs the assembly of FtsZ [63]. A 40-amino acid peptide, MciZ (mother cell inhibitor of FtsZ), is activated during the sporulation in *B. subtilis* cells and inhibits the assembly of FtsZ in a GTP-dependent manner [64]. Most of these proteins interact at the extreme C-terminal tail region of FtsZ, with some exceptions such as MciZ and SulA. For example, MciZ has been predicted to bind near the nucleotide-binding site on FtsZ and SulA binds to the T7 loop surface of FtsZ [64, 65]. The role of these accessory proteins in modulating FtsZ assembly dynamics and their mechanisms of regulation have been studied extensively but still a great deal of knowledge about them is required in order to get a complete understanding about their functions.

7.2.5 Proteins Involved in Septum Formation

The formation of the Z-ring is followed by the assembly of divisome apparatus, a well-concerted event involving the interplay of at least 10 essential proteins, which mediates septum synthesis and eventually leads to bacterial cytokinesis [66]. The interaction of the proteins involved occurs through diffusion and capture, whereby proteins diffuse from their origin to the specific location and are captured by their interacting partners [67]. It is suggested that these proteins are recruited in a hierarchical manner on the basis of their topological functions. The recruitment of these proteins to the division site depends on the presence of upstream components [68]. Initially, FtsZ assembles at the mid cell with the aid of FtsA and ZipA to form a proto-ring [69]. The proto-ring forms a scaffold for the assembly of other essential components of the divisome apparatus. The first protein to be recruited at the proto-ring is FtsK, which requires the presence of FtsZ, FtsA, and ZipA [70]. The next step involves the localization of three proteins, namely, FtsQ/DivIB, FtsL, and FtsB/DivIC, which are recruited as FtsQLB complex [71]. Proteins responsible for peptidoglycan synthesis are next to follow, with FtsW, a precursor transporter, and FtsI/PBP3, a septation-specific transpeptidse, being added to the divisome [72, 73]. Subsequently, FtsN arrives and provides the structural and functional integrity to the entire complex, nearly completing the divisome assembly [74]. FtsN has also been predicted to play a role in triggering the constriction process during cell division [75]. Finally, two proteins, AmiC and EnvC, that hydrolyze the septal murein and contribute to the splitting of cellular septum are known to be recruited [76, 77].

7.2.6 Role of Other Cytoskeleton Proteins in Bacterial Cell Division

The regulation of the cellular organization in bacteria is primarily dependent on the major cytoskeletal elements, FtsZ, MreB, and the intermediate filaments [7, 78, 79]. These proteins assist in the spatial as well as temporal localization of the various cellular components and act as mediators for the association of other cell-division-related proteins. In addition, these factors generate constriction forces required for the cell to divide and also contribute to the structural integrity of the cells. Another important feature of these cytoskeletal elements is that these proteins polymerize to form filaments and the interaction of these filamentous structures with other cellular components ensures the proper functioning of the cell. As FtsZ has been discussed in detail, in this section we stress on other bacterial cytoskeletal elements.

The actin homolog in bacteria, MreB, is an essential protein for maintaining the shape of the cell [78]. In *E. coli*, it has been found that the inhibition of MreB function leads to the loss of the rod shape of the cells and produces round-shaped cells [80]. MreB has been reported to have essential roles in several other cellular processes such as maintenance of cell polarity, localization of other cell-division-related proteins, the dynamicity associated with chromosomes, and in the regulation of virulence factors [81]. Further, MreB in association with proteins such as MreC, MreD, penicillin-binding protein 2 (PBP2), RodA, RodZ, and MurG, initiates the formation of the cell wall at a new position during the division process [81]. *In vitro* studies with *Thermatoga maritima* MreB have shown that MreB undergoes polymerization in the presence of ATP or GTP to form double-stranded filaments [82, 83]. In some bacterial species such as *B. subtilis*, homologs of MreB have been found to coexist and act in association with each other to maintain the shape of the cell [84, 85]. In addition, ParM proteins and ParA-type proteins regulate the process of plasmid segregation in several bacteria [86–88]. ParM is also a homolog of actin and encoded by the bacterial plasmids [86].

The third major cytoskeletal protein, crescentin, found in *Caulobacter* cells, has resemblance to the mammalian intermediate filament proteins [79]. Unlike FtsZ and MreB, crescentins form filamentous structures *in vitro* in the absence of nucleotides and contribute to maintaining the curvature of the *Caulobacter* cells. Although crescentins lack dynamicity *in vivo*, they have been found to interact with other cell division proteins [89]. MreB and a cytoskeletal metabolic enzyme, CtpS, have been known to inhibit the localization and assembly of crescentins [90]. In addition, other cytoskeletal proteins such as the bacterial dynamin-like protein (BDLP) have been identified in some bacterial species. These are GTPases, which are found to assemble as tubules and vesicles and contribute in maintaining the membrane structure, although their function is yet to be fully understood [91]. In addition, there are various other proteins such as bacterial dynamin, fibril proteins, and MreB-like filaments that are part of the cytoskeleton in different bacteria [92–94].

7.3
Cell Division Proteins as Therapeutic Targets

Designing inhibitors targeting the cell division process in bacteria has gained profound importance in recent years. An inhibitor that has the ability to restrain the proliferation of bacterial cells is supposed to have an immense potential to be developed as an antibacterial drug. As mentioned earlier, the cell division process involves an array of proteins and most of them are indispensable for the successful division of the cell. FtsZ, being the central and most studied one, has received more attention for drug development purposes. Nonetheless, the other accessory proteins also provide a vast scope for exploring them as therapeutic targets. As the cell division machinery is a largely unexplored antibacterial drug target, working toward this direction might produce a good dividend.

7.3.1
FtsZ as a Therapeutic Target

FtsZ is the chief protein involved in the cell division of bacteria. Its functional importance in facilitating the division of the cell has led to its recognition as one of the major antibacterial drug targets. Moreover, the key features such as its wide conservation in bacteria [4], constant intracellular concentration throughout the bacterial cell cycle [95], and clear structural and functional characterization make it all the more a suitable candidate for drug development. Several antitubulin drugs have been actively pursued as anticancer agents in leukemia and other types of cancers such as breast, lung, cervical, and ovarian [96–98]; therefore, it is logical to think that anti-FtsZ agents may also be successfully used as antibacterial drugs. In the past few years, various FtsZ-targeting compounds have been identified, which can act as potential lead compounds for developing effective antibacterial drugs. FtsZ-targeting antibacterial agents can act either as depolymerizing agents/disassembly inducers or polymerizing agents/assembly promoters. Both of these classes of agents are significant because both polymerization and depolymerization of FtsZ are imperative for the successful completion of bacterial cytokinesis. In this section, we explain the approaches that can be undertaken for the identification of FtsZ inhibitors and provide a brief description of several FtsZ-targeting antibacterial agents.

7.3.1.1 **Identification of FtsZ-Targeting Antibacterial Agents**

FtsZ-targeted agents can be effectively screened on the basis of their effects on FtsZ assembly. Inhibitors of FtsZ assembly have either been derived from the natural products or have been identified from libraries of semisynthetic and synthetic compounds. Identification of an FtsZ-targeting compound can be achieved by various biochemical and/or cellular studies. Exploiting different assembly properties of FtsZ and its inhibition phenotypes, several simple assays have been devised for large-scale screening of FtsZ-targeting compounds. These include FtsZ assembly and GTPase activity *in vitro* and various cell-based assays.

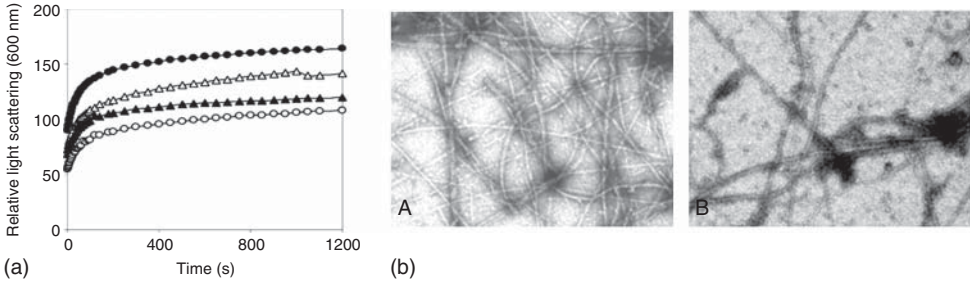


Figure 7.2 (a) Shown are the light scattering traces of *E. coli* FtsZ in the absence (●) and presence of 25 (△), 50 (▲), and 100 μM (◯) sanguinarine. *Source*: Reprinted with permission from Ref. [101]. Copyright (2005) American Chemical Society. (b) Electron micrographs showing the effects of totarol on the morphology of *Mtb*FtsZ polymers. (A,B) show FtsZ polymers in the absence and presence of 25 μM totarol, respectively. Scale bar is 500 nm. *Source*: Reprinted with permission from Ref. [102]. Copyright (2007) American Chemical Society.

As FtsZ monomers easily assemble to form polymers *in vitro* under suitable conditions, the effect of a compound on the assembly of FtsZ can be conveniently examined outside the bacterial cells using methods such as light scattering, sedimentation assay, and transmission electron microscopy. For instance, the decrease in light scattering intensities in the presence of increasing concentrations of sanguinarine indicated that sanguinarine decreases the polymerization of *E. coli* FtsZ (Figure 7.2). The rhodanine series of compounds were screened by determining their effects on the assembly of *E. coli* FtsZ using the sedimentation assay [99]. Electron microscopic analysis of the assembly of FtsZ indicated that totarol inhibits the assembly of *Mycobacterium tuberculosis* FtsZ (*Mtb*FtsZ) (Figure 7.2). Further, the GTPase activity of FtsZ is essential for the functioning of FtsZ; therefore, an analysis of the effect of the compound on the GTPase activity of FtsZ is included as either a primary or one of the important steps in the screening procedure [100].

In addition, the interaction of the compound with purified FtsZ can be monitored by binding assays. A change in the spectral properties of either the compound or the protein upon binding may be used to estimate the binding constant. For example, if the compound exhibits fluorescence, then the change in the fluorescence intensity of the compound in the presence of the protein may be used to determine its binding to FtsZ. The interaction between curcumin and FtsZ was monitored by following the increase in curcumin fluorescence upon binding to FtsZ and the dissociation constant (K_d) of 7.3 ± 1.8 μM was estimated from the fluorescence data [103]. Alternatively, a tryptophan residue may be introduced in FtsZ by site-directed mutagenesis as the native FtsZ does not contain a tryptophan residue and the change in the intrinsic fluorescence of the mutated FtsZ can be used to determine the interaction with an inhibitor. For example, the Y371W mutant was constructed to introduce a tryptophan residue in *E. coli* FtsZ [101]. Sanguinarine was found to reduce the intrinsic tryptophan fluorescence intensity of Y371W-FtsZ in a concentration-dependent manner. From the double reciprocal

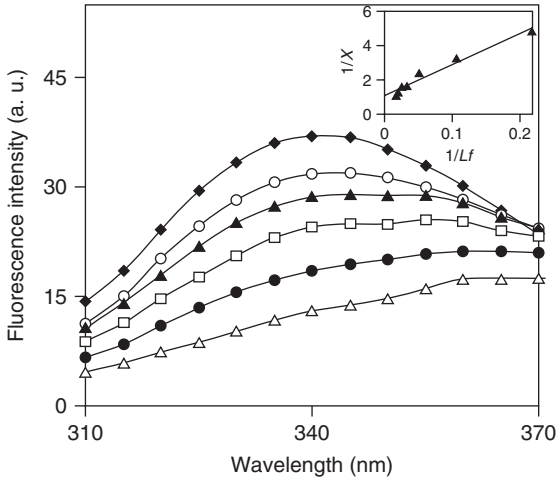


Figure 7.3 The tryptophan emission spectra in the absence (♦) and presence of 5 μM (○), 10 μM (▲), 20 μM (□), 40 μM (●), and 60 μM (Δ) sanguinarine has been shown. The excitation and emission wavelengths were 295 and 340 nm, respectively. The inset shows a double-reciprocal plot where the reciprocal of free ligand concentration (*Lf*) has been plotted against the reciprocal of bound ligand concentration (*X*). *Lf* and *X* have been calculated using the formula $Lf = C - X[Y]$ and $X = (F_o - F)/\Delta F_{max}$, respectively. The dissociation constant has been calculated using the relation $1/X = 1 + K_d/Lf$. *Source*: Reprinted with permission from Ref. [101]. Copyright (2005) American Chemical Society.

plot shown in Figure 7.3, a K_d of the interaction between FtsZ and sanguinarine was estimated to be 18.4 ± 1.6 μM. In addition, FtsZ can be labeled with an external fluorophore (fluorescein isothiocyanate (FITC), acrylodan, or alexa dyes), and the change in its fluorescence can be monitored upon ligand binding. Sophisticated techniques such as isothermal titration calorimetry (ITC) and nuclear magnetic resonance (NMR) spectroscopy can also be employed to monitor the interaction between FtsZ and small molecules *in vitro* [104–107].

A defect in FtsZ assembly has been shown to increase bacterial cell length [101, 102]; thus, an inhibitor of FtsZ is expected to increase the length of bacterial cells. The elongation of the cell occurs either because of the lack of Z-ring formation or the formation of nonfunctional Z-ring, which is unable to constrict and is thus incompetent in carrying out cell division. Inhibitors of FtsZ have been found to strongly increase bacterial cell length. For example, treatment with 1.5 μM totarol increased *B. subtilis* cell length from 3.9 ± 1.0 to 22.4 ± 14.8 μm (Figure 7.4; [106]). Next, the effect of the inhibitor on the status of the Z-ring inside the bacterial cells is examined. The cells treated with the compound of interest can be stained using FtsZ antibody to visualize the Z-ring (Figure 7.4; [98, 101, 106]). Alternatively, GFP-tagged FtsZ is expressed in bacterial cells and the localization of FtsZ is monitored in compound treated cells [101, 108]. The latter technique also facilitates the monitoring of FtsZ dynamics in live bacterial cells. Once the compound is found to perturb FtsZ assembly, the antibacterial activity of the compound is

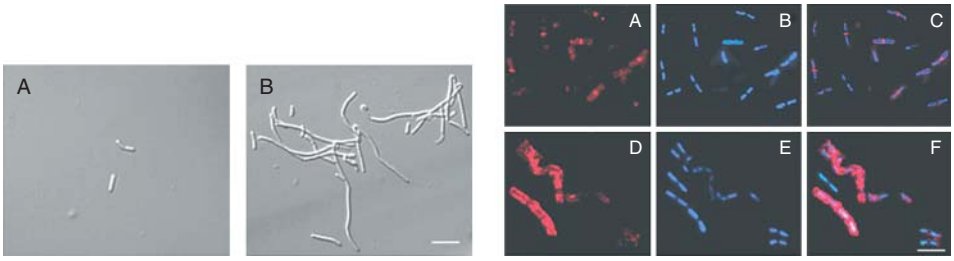


Figure 7.4 Totarol treatment induced filamentation and inhibited the formation of the Z-ring in *B. subtilis* 168 cells. (a) DIC images of *B. subtilis* 168 cells without (A) and with totarol treatment (B). (b) Fluorescence microscopy images of *B. subtilis* 168 cells stained for FtsZ (red) and DNA (blue). (A–C) show control cells and (D–F) show totarol-treated cells. *Source*: Reprinted with permission from Ref. [102]. Copyright (2007) American Chemical Society.

determined by estimating its half-maximal inhibitory concentration (IC_{50}) value or minimal inhibitory concentration (MIC).

This is a broad summary of the experiments that are generally performed for the screening of FtsZ inhibitors. The order and the number of experiments for screening are flexible and might vary in different studies. Once it is established that FtsZ is the target of an inhibitor, the putative binding site of the inhibitor on FtsZ may be identified using docking studies and then the mutants of FtsZ are constructed to validate the putative interactions [108]. Knowledge of the mode of the interaction of an inhibitor with FtsZ is of immense importance as it would further help in structure-based drug designing. It is important to check the toxicity of the inhibitor against mammalian cells as FtsZ and tubulin are homologs and a molecule active against FtsZ may also bind to tubulin. The more the difference in the IC_{50} values of an inhibitor in inhibiting bacterial and mammalian cell proliferation, the greater are the chances that the inhibitor might not be toxic to the host. An FtsZ inhibitor with a substantially low MIC value could be considered for testing on animal models, which further qualifies it for clinical trials.

7.3.1.2 FtsZ Inhibitors

The following is a brief description of small molecules that inhibit bacterial cell division by affecting the assembly properties of FtsZ.

2-Alkoxycarbonylaminopyridines These compounds were screened for their antibacterial activity against the growth of *M. tuberculosis* H37Rv [109]. Two compounds in this group, ethyl {8-[[4-(diethylamino)-1-methylbutyl]-amino]-2,3-diphenylpyrido(2,3-b)pyrazin-6-yl}carbamate (SRI-3072) (Figure 7.5) and ethyl [6-amino-2,3-dihydro-4-phenyl-1H-pyrido(4,3-b)(1,4)diazepin-8-yl]carbamate (SRI-7614) (Figure 7.5) proved to be very effective in inhibiting the growth of *M. tuberculosis* H37Rv. These compounds inhibited the growth of *M. tuberculosis* with MIC_{99} (minimum concentration of compound causing 99% inhibition) of

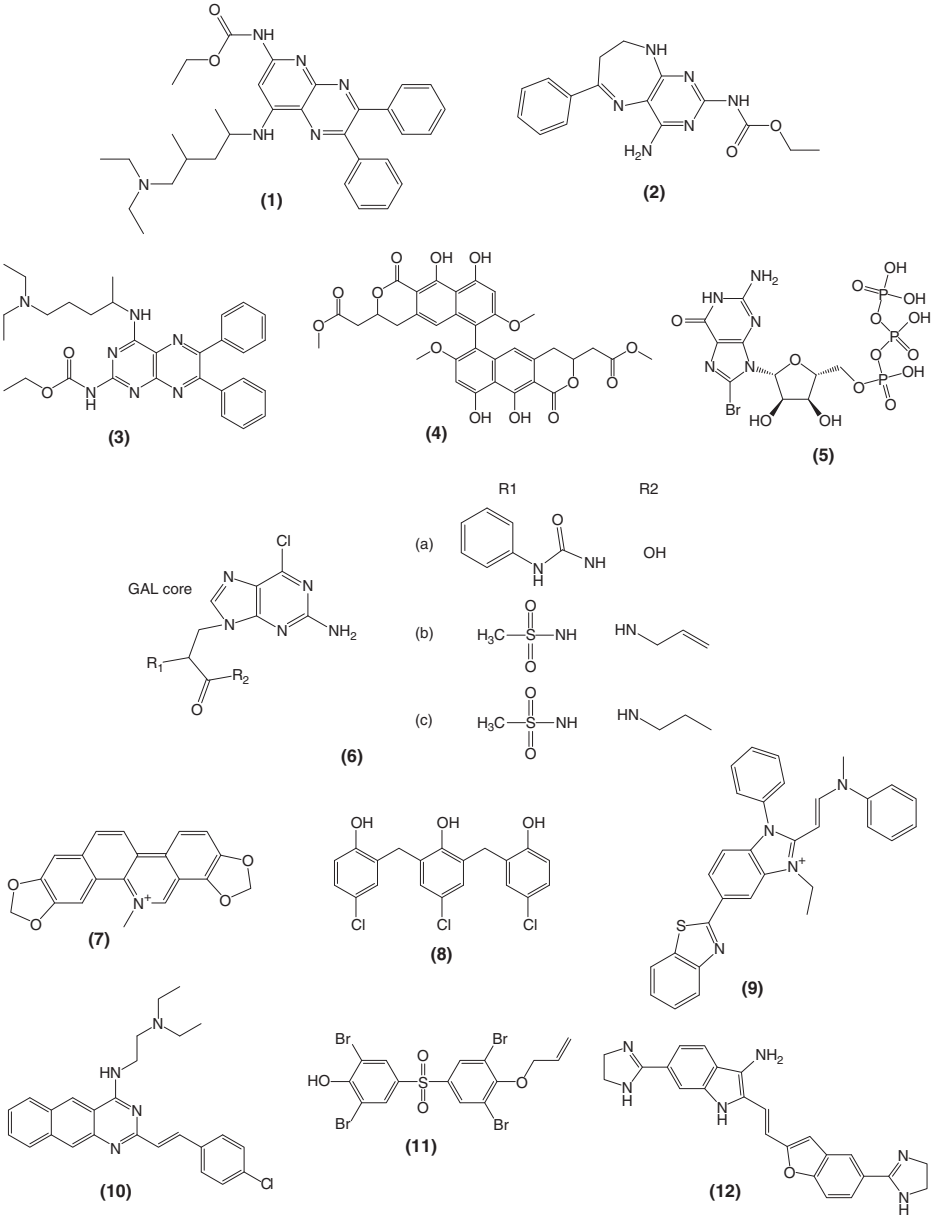
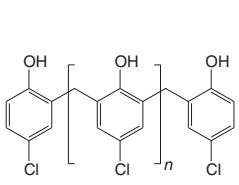


Figure 7.5 Structures of FtsZ inhibitors – (**1**) SRI-3072, (**2**) SRI-7614, (**3**) 2-carbamoyl-pteridine, (**4**) viriditoxin, (**5**) 8-bromoguanosine 5′-triphosphate, (**6**) GAL analogs (a–c are for three different analogs), (**7**) sanguinarine, (**8**) Z1, (**9**) Z2, (**10**) Z3, (**11**) Z4, (**12**) Z5, (**13**) nZ1 (oligochlorophens), (**14**) dichamentin, (**15**) 2″-hydroxy-5″-benzylisouvarinol-B, (**16**) curcumin, **17**) TRA 10a, (**18**) totarol, (**19**) berberine, (**20**) OTBA, (**21**) PC190723, (**22**) 8j, (**23**) benzimidazole (1a-G1), (**24**) chrysophaentin A, and (**25**) chlorogenic acid.



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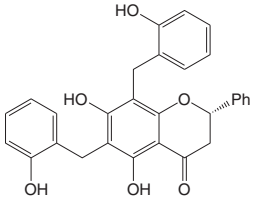

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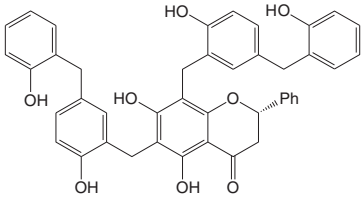

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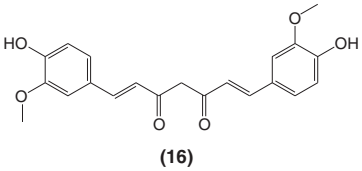

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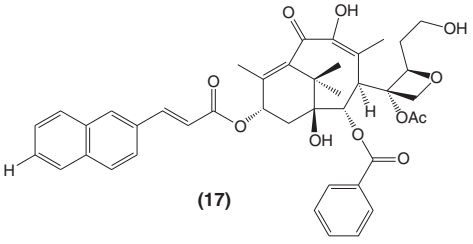

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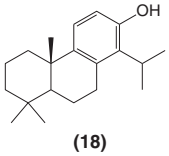

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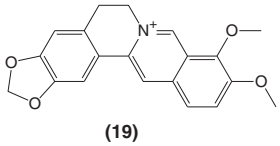

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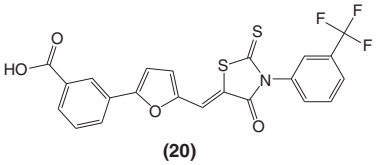

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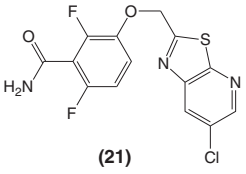

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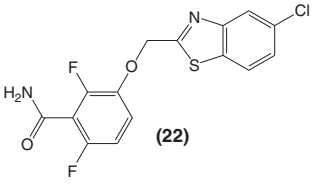

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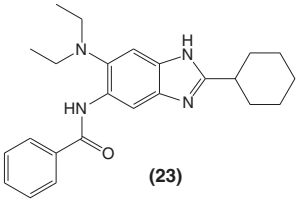

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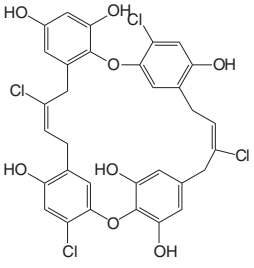

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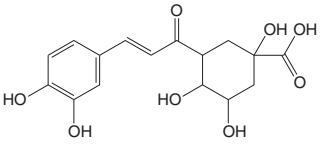

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Figure 7.5 (*Continued*)

0.15 (SRI-3072) and 6.25 mg l^{-1} (SRI-7614). In addition, SRI-3072 and SRI-7614 inhibited the polymerization of purified *M. tuberculosis* FtsZ by 50% at 52 ± 12 and $60 \pm 0\,\mu M$, respectively. Both compounds were also effective against the *M. tuberculosis* strains resistant against ethambutol, rifampicin, kanamycin, isoniazid, cycloserine, and pyrazinamide. SRI-3072 was even found to decrease the growth of *M. tuberculosis* Erdman in macrophages, qualifying it as a promising antitubercular agent [109].

In a follow-up study, two compounds from the above-mentioned library were used as lead compounds to synthesize potent pyridopyrazine and pyrimidothiazine derivatives [110]. The MIC of these compounds was tested for *M. tuberculosis* H37Ra and *M. tuberculosis* H37Rv, and a few derivatives were found to have MIC lower than the parent compounds.

2-Carbamoyl Pteridine This compound was identified from a library of synthetic compounds initially designed against malarial parasites. A set of compounds having structural similarities with known antitubulin agents was selected from this library and tested for their antibacterial activities [111]. 2-Carbamoyl pteridine (Figure 7.5) inhibited the polymerization of *Mtb*FtsZ by 50% at $34.2 \pm 2.5\,\mu M$ and also suppressed its GTPase activity by 35% at 100 μM. Interestingly, the compound had no effect on tubulin polymerization at the same concentration. However, a derivative of 2-carbamoyl pteridine, 2,4,6,7-tetrasubstituted pteridine, was found to be less potent in inhibiting *Mtb*FtsZ polymerization as compared to the parent compound.

Viriditoxin This compound was identified amongst >100 000 extracts of microbial fermentation broths and plant extracts for its anti-FtsZ activity [112]. In one of the active extracts, viriditoxin (Figure 7.5) was found to be the compound responsible for the antibacterial activity. The polymerization of FtsZT65C-fluorescein was monitored in the presence of purified viriditoxin. The polymerization of FtsZ was found to be inhibited by 50% in the presence of $8.2\,\mu g\,ml^{-1}$ viriditoxin. Viriditoxin also inhibited the GTPase activity of FtsZ with an average IC_{50} value of $7.0\,\mu g\,ml^{-1}$. Moreover, it induced elongation in bacterial cells, which is indicative of FtsZ inhibition. Viriditoxin was found to be active against several multidrug-resistant gram-positive pathogenic bacteria including *Streptococcus*, *Enterococcus*, and *Staphylococcus*, with the MIC value ranging between 2 and 32 $\mu g\,ml^{-1}$ [112].

GTP Analogs A GTP analog, 8-bromoguanosine 5′-triphosphate (Figure 7.5), was designed by modifying GTP with the idea of inhibiting FtsZ assembly [113]. The mode of binding of GTP to FtsZ and tubulin was analyzed and the similarities and differences between its interactions with these proteins were identified. The analysis suggested that a C8-substituted GTP analog might not affect its binding to FtsZ but would probably block the interaction with the next monomer. Thus, 8-bromoguanosine 5′-triphosphate was synthesized and its effect on FtsZ polymerization was checked. It did not support the polymerization of FtsZ; moreover, it competitively inhibited the polymerization and GTPase activity of FtsZ when added

along with GTP. Notably, 8-bromoguanosine 5′-triphosphate did not perturb the polymerization of tubulin [113].

Paradis-Bleau *et al*. [114] also synthesized a series of GTP analogs, referred to as *GAL analogs*. These analogs had a guanine group of GTP replaced with a guanine-like moiety linked to an alanine side chain. The GAL analogs (Figure 7.5) acted as irreversible inhibitors and inhibited the GTPase activity of *Pseudomonas aeruginosa* FtsZ, with IC_{50} values ranging from 450 μM to 2.6 mM. Among the nine compounds synthesized, three compounds were found to be potent against *Staphylococcus aureus* proliferation.

Sanguinarine This is a natural compound obtained from the roots of the herbaceous plant *Sanguinaria canadensis*. Sanguinarine (Figure 7.5) has long been known for its antibacterial activities [115, 116]. It also possesses activity against cancer cells [117, 118]. Sanguinarine has been reported to inhibit the proliferation of *B. subtilis* 168, *E. coli* BL21, and *E. coli* JM109 (WM647) cells with IC_{50} values of 3 ± 1, 14 ± 2.3, and 36 ± 5.1 μM, respectively [101]. Furthermore, it has been shown to be active against the methicillin-resistant *S. aureus* (MRSA) [119]. The MIC for two strains of *S. aureus* was 3.12 and 1.56 μg ml^{-1}, respectively. Beuria *et al*. [101] suggested that sanguinarine inhibits bacterial proliferation by targeting the assembly of FtsZ. *In vitro*, sanguinarine inhibited the assembly and bundling of purified FtsZ. It bound to FtsZ (Y371W) with a K_d of 18.4 ± 1.6 μM. Sanguinarine was found to strongly increase bacterial cell length; for example, 5 μM sanguinarine induced a fivefold increase in *B. subtilis* 168 cell length, and 18 μM sanguinarine increased the cell length of *E. coli* BL21 by eightfold as compared to the vehicle-treated cells [101]. In addition, sanguinarine perturbed the formation of Z-rings in *B. subtilis* 168 cells; the frequency of Z-ring occurrence per μm cell length in *B. subtilis* 168 cells was found to be 0.22 ± 0.02 and 0.02 ± 0.02 in the absence and presence of 8 μM sanguinarine, respectively [101]. However, the frequency of occurrence of nucleoids per micrometer of the cell length was found to be unaltered upon sanguinarine treatment, suggesting that sanguinarine had no effect on the nucleoid segregation. Similarly, sanguinarine was found to perturb the Z-ring formation in *E. coli* JM109 cells expressing GFP-FtsZ [101]. Finally, the membrane integrity of *E. coli* cells was found to be unaffected in the presence of sanguinarine.

Zantrins A large library of compounds was screened to find FtsZ inhibitors by monitoring their effects on the GTPase activity of FtsZ [100]. Five compounds that significantly decreased the GTPase activity of the *E. coli* FtsZ (∼50% at <50μM) were selected. These compounds were named Zantrins (Fts*Z* gu*a*no*s*ine *t*ri*p*hosphatase *in*hibitors; Z1–Z5 shown in Figure 7.5, respectively) as it symbolizes their effects on FtsZ. Z1, Z2, and Z4 caused a decrease in FtsZ polymers, whereas Z3 and Z4 increased the amount of polymeric FtsZ as compared to the control. The effects of these Zantrins were also checked on gram-negative and gram-positive pathogenic bacteria. Zantrins (Z1–Z5) were found to be effective against *Shigella dysenteriae* 60R, *Vibrio cholerae* strain N16961, *P. aeruginosa, Bacillus cereus, Streptococcus pneumoniae* type 4 TIGR4 strain, although the effects were differential.

For example, *S. dysenteriae* 60R and *V. cholerae* strain N16961 were found to be more effectively inhibited by Z1–Z3, whereas Z1 and Z4 inhibited *B. cereus* and *S. pneumoniae* type 4 TIGR4 efficiently.

Z1 inhibited the growth of *B. cereus* at a very low concentration (MIC = 0.625 μM). Therefore, Z1 and its oligomeric analogs (nZ1, oligochlorophens; Figure 7.5) were further explored for their antibacterial activities [120]. 4Z1 showed better antibacterial activities than 3Z1 (referred to as *Z1* earlier). It inhibited the growth of *B. cereus* with an MIC of 0.08 μM, approximately eightfold greater than that of 3Z1 and that of *Bacillus anthracis* Sterne 7702 with an MIC of 0.16 μM [120].

Dichamentin and 2‴-Hydroxy-5″-Benzylisouvarinol-B These are natural polyphenolic compounds isolated from the *Uvaria chamae* and *Xylopia afticana*, respectively. While 2″-hydroxy-5″-benzylisouvarinol-B (Figure 7.5) was found to inhibit the growth of a variety of bacteria including *E. coli*, *B. subtilis*, *M. smegmatis*, *S. aureus*, *and P. aeruginosa* within the MIC range of 3–16 μM, dichamentin (Figure 7.5) inhibited the growth of *B. subtilis*, *M. smegmatis*, and *S. aureus* at lower concentrations (2–4 μM) [121]. Dichamentin was not active against tested gram-negative bacteria viz, *E. coli* and *P. aeruginosa*. These compounds were synthesized and tested for their FtsZ-targeting abilities as they have structural similarities with zantrins (Z1), which were earlier seen to inhibit bacterial growth by targeting FtsZ. It was found that both of these compounds inhibited the GTPase activity of *E. coli* FtsZ, with the IC_{50} values 12.5 ± 0.5 and 8.3 ± 0.5, comparable to that of Z1 [121].

Curcumin (Diferuloylmethane) Curcumin (Figure 7.5) is a naturally occurring polyphenolic compound, which is obtained from the rhizomes of *Curcuma longa*. Turmeric (dried and grounded rhizomes of *C. longa*) has been used for therapeutic purposes for several years and the presence of curcumin has been attributed to the antibacterial properties of turmeric. Curcumin is believed to have multiple targets and FtsZ has been identified as one of its targets [103]. It was found that curcumin treatment inhibited the proliferation of *B. subtilis* 168 and *E. coli* K12 cells with IC_{50} values of 17 ± 3 and 58 ± 5 μM, respectively. Further, curcumin induced filamentation in *B. subtilis* 168 cells and perturbed Z-ring formation in these cells. It also reduced the assembly and bundling of FtsZ filaments, and increased the GTPase activity of FtsZ. *In vitro*, curcumin bound to FtsZ with a K_d of 7.3 ± 1.8 μM and the binding of curcumin altered the secondary structure of FtsZ [103]. The results indicated that curcumin targeted FtsZ by perturbing its assembly dynamics.

Taxanes Taxanes are well known for their anticancer activity. They inhibit the growth of cancer cell lines by targeting microtubules in eukaryotic cells. Huang *et al.* [122] examined 120 taxanes for their activity against *M. tuberculosis* and by using microdilution broth assay found several promising inhibitors among these compounds. Two of these inhibitors caused elongation of the cells. One of these inhibitors, TRA 2a, was selected and derivatives were synthesized by substitution

to reduce its cytotoxicity, improve water solubility, and increase potency against *M. tuberculosis*. Finally, TRA 10a (Figure 7.5) and three analogs were obtained, which had improved MIC (1.25–2.5 μM) against *M. tuberculosis* strains H37Rv and IMCJ946K2, and much reduced cytotoxicity in cancer cell lines ($IC_{50} > 80\,\mu M$ for MCF-7 and A549 cells).

Totarol Totarol (Figure 7.5), a diterpenoid phenol, is extracted from *Podocarpus totara*. The antibacterial activities of totarol are well established. It has been shown to inhibit the growth of a number of gram-positive bacteria such as *S. aureus, Propionibacterium acnes*, and *M. tuberculosis* [123, 124]. It has been shown to target FtsZ in bacterial cells [102]. Totarol inhibited the growth of *B. subtilis* 168 cells with MIC of 2 μM. It induced filamentation in *B. subtilis* cells and perturbed the Z-ring formation but did not disrupt membrane structure or nucleoid segregation. *In vitro*, totarol inhibited the assembly of purified *Mtb*FtsZ and also decreased the GTPase activity of *Mtb*FtsZ. Furthermore, totarol bound to *Mtb*FtsZ with a K_d of $11 \pm 2.3\,\mu M$. Moreover, in a mammalian cancer cell line (HeLa), its IC_{50} has been found to be $\sim 18\,\mu M$, indicating its specificity toward bacterial cells. It is a potential lead compound as it effectively and specifically targets FtsZ [102]. Recently, synthesis of totarol and its related compounds totaradiol and totarolone have been reported [125], which may help to study the mechanism of FtsZ inhibition by these compounds.

Berberine Berberine (Figure 7.5) is known to be active against a wide species of bacteria including both gram-negative and gram-positive bacteria [126, 127]. It has also been found to possess activity against certain drug-resistant species of pathogenic bacteria such as *M. tuberculosis* and *S. aureus* [128, 129]. Berberine is shown to bind to DNA *in vitro* [130, 131]; however, the prime target for its antibacterial activity has remained ambiguous. In a recent study, berberine has been found to inhibit the assembly of FtsZ [132]. It has been reported to inhibit the assembly and GTPase activity of FtsZ with IC_{50} values of 10 ± 2.5 and $16 \pm 5.0\,\mu M$, respectively. A K_d value for FtsZ and berberine has been determined to be $\sim 0.02\,\mu M$ using ITC. Furthermore, saturation transfer difference-nuclear magnetic resonance (STD-NMR) and molecular docking studies have revealed that the binding site of berberine overlaps with the GTP-binding site on FtsZ. Berberine treatment also perturbs Z-ring formation in the *E. coli* cells [132]. In a separate study, a genetic approach was used to verify FtsZ as a target for berberine [133]. Silencing of *ftsZ* in *E. coli* led to its sensitization to berberine treatment and the overexpression of FtsZ circumvented the toxic effects of berberine in *E. coli*, suggesting that berberine targets FtsZ in the bacterial cells.

OTBA OTBA (3-{5-[4-oxo-2-thioxo-3-(3-trifluoromethyl-phenyl)-thiazolidin-5-yli-denemethyl]-furan-2-yl}-benzoic acid; Figure 7.5) was selected from a library of 81 rhodanine derivatives [99]. The compounds were screened on the basis of their effect on the polymerization of purified *E. coli* FtsZ as determined by the sedimentation assay. On the basis of change in polymer mass, 12 compounds

(9 decreased polymer level by 15–25%, 2 decreased polymer level by 30–35%, and 1 increased polymer level by 25–30%) were selected. The effect of these 12 compounds was then monitored on *B. subtilis* 168 cell proliferation. One of the compounds, OTBA, that increased the assembly of FtsZ and also potently inhibited the proliferation of *B. subtilis* cells, was selected for further studies. OTBA was found to inhibit the proliferation of the *B. subtilis* 168 cell with an MIC of 2 μM; it induced filamentation and also perturbed Z-ring formation in these cells. *In vitro*, it promoted the assembly and bundling of both *E. coli* and *B. subtilis* FtsZs as evident from light scattering, electron microscopy, and fluorescence microscopic experiments. FtsZ polymers are known to disassemble upon dilution because of the lowering of monomer concentration in solution. OTBA prevented the dilution-induced disassembly of FtsZ polymers, indicating that it is an FtsZ-stabilizing agent. In addition, it reduced the GTPase activity of both *E. coli* and *B. subtilis* FtsZ. For example, the rate of GTP hydrolysis of *E. coli* FtsZ was reduced by ~60% in the presence of 50 μM OTBA and that of *B. subtilis* FtsZ by ~48% in the presence of 40 μM OTBA. The interaction of OTBA and *E. coli* FtsZ was monitored using the tryptophan fluorescence of a functionally active mutant, Y371W. OTBA decreased the tryptophan fluorescence of *E. coli* FtsZ in a concentration-dependent manner and the dissociation constant of the interaction was observed to be 15 ± 1.5 μM. Further, OTBA inhibited the proliferation of HeLa cells with an IC_{50} of ~8 μM and it did not alter microtubule organization in cells. In addition, OTBA did not affect microtubule polymerization *in vitro*, suggesting that OTBA was more effective against bacterial cells as compared to mammalian cells [99]. OTBA can act as a structural scaffold for the development of potent FtsZ inhibitors. This can be achieved through the molecular understanding of its interaction with FtsZ and then synthesizing the derivatives accordingly.

PC190723 and 8j PC190723 ($C_{14}H_8ClF_2N_3O_2S$) (Figure 7.5) was identified from a library of compounds specially designed to target FtsZ [108]. The parent compound used was 3-methoxybenzoic acid (3-MBA), which had earlier been reported to bind to FtsZ, albeit weakly [134]. The methoxy group of 3-MBA was substituted by various groups and then the derived compounds were tested for their antibacterial efficacy. The MIC of PC190723 was found to be 1 μg ml^{-1} for *B. subtilis* and *Staphylococcus* species including *S. aureus*, MRSA, multidrug-resistant *S. aureus* (MDRSA), *S. epidermidis, S. haemolyticus, S. hominis, S. lugdunensis, S. saprophyticus, S. warneri*. It caused the elongation/enlargement of the cells accompanied by mislocalization of FtsZ in cells [108]. Experiments with purified FtsZ indicated that it stabilized FtsZ polymers and decreased the GTPase activity of FtsZ [135]. The compound was also effective when tested in mouse models infected with *S. aureus* with the ED_{50} (effective dose 50%) values of 7.3 and 10.2 mg kg^{-1}, when the compound was administered subcutaneously and intravenously, respectively [108]. Effects of one more analog of PC190723 from this library, namely, 8j (Figure 7.5) were studied on FtsZ [136]. 8j stabilized FtsZ polymers as observed in light scattering and sedimentation assay and also inhibited the GTPase activity of FtsZ. The FtsZ polymers became highly curved after treatment with 8j. Cytological effects of 8j

were monitored using GFP-FtsZ-transformed *Bacillus* cells. 8j treatment was found to cause mislocalization of FtsZ [136]. Also, the effects of 8j were monitored in cells with preformed FtsZ ring using FRAP assay. It was found that 8j interfered with FtsZ dynamics in the Z-ring as the recovery time of GFP-FtsZ in the Z-ring increased significantly in 8j-treated cells after bleaching GFP-FtsZ from the Z-ring. In addition, the localization patterns of several downstream proteins of FtsZ, such as FtsA, ZapA, EzrA, SepF, FtsL, DivIC, PBP2B, and FtsW in 8j-treated cells were found to be different from that of the control cells [136]. The findings indicated that benzamide compounds could be important as FtsZ-targeting antibacterial agents.

Benzimidazoles A library of benzimidazole compounds was synthesized and tested against *M. tuberculosis* H37Rv [137]. These were 2,5,6- and 2,5,7-trisubstituted benzimidazoles designed with a view to develop novel FtsZ-targeting compounds. Among the 349 compounds synthesized, around 26 were found to have MIC $\leq 5\,\mu g\,ml^{-1}$ for *M. tuberculosis* H37Rv strain as determined by microplate alamar blue assay (MABA). As cyclohexyl and diethylamino groups at 2- and 6-positions were thought to be important for the antibacterial activity of these compounds, a new library of compounds (designated as 1-G) was synthesized by substituting diethylamino groups at the 6-position. Out of the 238 compounds generated, 5 compounds (1a-G4, 1a-G7, 1b-G1, 1b-G2, and 2b-1) were tested for their activity against the drug-resistant strains of *M. tuberculosis* W210, NHN20, NHN335-2 (isoniazide-resistant, KasA G269S mutation), NHN382 (isoniazide-resistant, KatG 315T mutation), and TN587 (isoniazide-resistant, KatG S315T mutation). The MIC value for these strains was in the range of 1–4.6 μM, as in the case of drug-sensitive strains. Moreover, three compounds (1a-G1 (Figure 7.5), 1a-G4, and 1a-G7) were tested for their effect on FtsZ polymerization and they efficiently inhibited FtsZ assembly and also altered its GTPase activity. Some of the promising compounds have been subjected to *in vivo* testing.

Chrysophaentins Chrysophaentins A–H (bisdiarylbutene macrocycles) have been isolated from the marine alga *Chrysophaeum taylori* [107]. The antibacterial efficacy of the compounds was examined using pathogenic bacteria, *S. aureus*, MRSA, *Enterococcus faecium*, and vancomycin-resistant *E. faecium*. Chrysophaentin A (Figure 7.5) was found to be the most effective of the eight compounds with MIC_{50} (minimum concentration causing 50% inhibition) value in the range of $1.5–4\,\mu g\,ml^{-1}$ for these bacteria [107]. Chrysophaentin A inhibited the assembly and GTPase activity of purified *E. coli* FtsZ. The IC_{50} for inhibition of GTPase activity was found to be $6.7 \pm 1.7\,\mu g\,ml^{-1}$. STD-NMR and molecular docking showed that chrysophaentin A bound with FtsZ at the GTP-binding site. In addition, chrysophaentin A did not inhibit growth of two cancer cell lines, namely, HCT-11 and P388 in culture even at the concentration of $50\,\mu g\,ml^{-1}$ and it had no effect on the polymerization of tubulin even at 150 μM, showing its specificity toward FtsZ. Chrysophaentins A–H are a new class of natural FtsZ-targeting compounds and seem to be promising antibacterial agents.

Phenylpropanoids These include the polyphenols, which are generally found in food products. Eight such phenylpropanoids (cinnamic, p-coumaric, caffeic, chlorogenic, ferulic, 3,4-dimethoxycinnamic, 2,4,5-trimethoxycinnamic acids, and eugenol) were tested for their effects on FtsZ [138]. Some of these compounds such as cinnamic acid, p-coumaric acid, caffeic acid, chlorogenic acid (Figure 7.5), eugenol, and ferulic acid are already known to possess antibacterial activity [139]. As part of the initial screening, the effect of these compounds was checked on the GTPase activity of purified *E. coli* FtsZ. All eight phenylpropanoids inhibited the GTPase activity of FtsZ; however, chlorogenic acid was found to be the most effective [139]. At 100 μM, it inhibited GTPase activity by 46% compared to the control. It inhibited FtsZ polymerization with an IC_{50} value of 70 μM. A docking analysis indicated that these compounds may bind to FtsZ near the T7 loop of FtsZ, which plays an important role in GTP hydrolysis. Furthermore, chlorogenic acid induced filamentation in *B. subtilis* 168 cells more efficiently than the other phenylpropanoids.

7.3.2
Other Cell Division Proteins as Therapeutic Targets

As explained, FtsZ is one of the major components of cell division machinery, which controls the fate of a bacterial cell. During cytokinesis, FtsZ forms a scaffold for the organization of other cell-division-related proteins and a concerted mechanism of action ensures the division of the cell. Although FtsZ remains to be the primary target for the development of antibacterial agents, exploiting the interaction of FtsZ with its accessory proteins may provide a lead for designing inhibitors of cell division. Inhibitors that hamper the interaction of FtsZ and its major accessory proteins may induce a significant defect in the divisome complex and inhibit the cell division. Thus, targeting the accessory proteins of the cytokinetic machinery may also be a highly rewarding exercise.

Among the proteins interacting directly with FtsZ, ZipA, and FtsA play very crucial roles. These are the positive regulators of FtsZ assembly and, being membrane-targeting proteins, they help in anchoring the Z-ring to the membrane. Various groups have synthesized compounds, using structure-based design, and combinatorial synthesis, to perturb FtsZ–ZipA interaction. Detailed knowledge of the FtsZ–ZipA interaction from the availability of the crystal structure of their complex [140] has facilitated these efforts. Sutherland *et al.* [141] constructed a series of chimeric compounds to inhibit the FtsZ–ZipA interaction. These chimeras were made by combining weak inhibitors, indoles, and oxazole compounds, yielding carboxybiphenyindoles (Figure 7.6) that displayed improved efficiency relative to the parent compounds. In addition, these chimeras were also found to inhibit the growth of several gram-positive and gram-negative bacteria [141]. Jennings *et al.* [142] reported a small molecule, 1,2,3,4,12,12b-hexahydro-indolo[2,3-a]quinolizin-7-one (Figure 7.6), which perturbed the interaction of ZipA and FtsZ with an IC_{50} of 1170 μM. Furthermore, a series

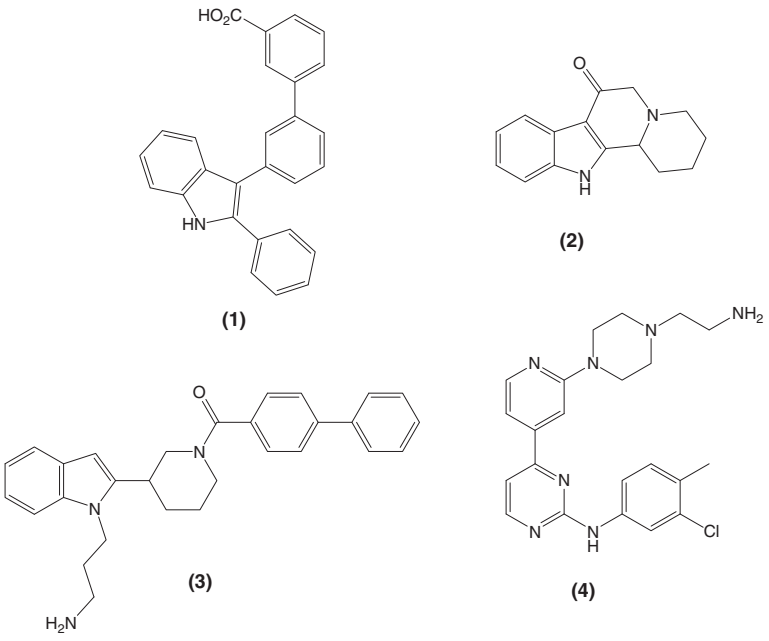

Figure 7.6 Structures of FtsZ–ZipA interaction inhibitors – (**1**) carboxybiphenylindole, (**2**) 1,2,3,4,12,12b-hexahydro-indolo[2,3-a]quinolizin-7-one, (**3**) {3-[1-(3-amino-propyl)-1*H*-indol-2-yl]-piperidin-1-yl}-biphenyl-4-yl-methanone (16.a.4), and (**4**) pyridylpyrimidine.

of compounds were synthesized by Jennings *et al.* [143] using the structure-based design approach to obtain the inhibitors of FtsZ–ZipA interaction. They obtained many compounds that bound to the FtsZ-binding region of ZipA, as determined by 2-D HSQC NMR. A compound, {3-[1-(3-amino-propyl)-1*H*-indol-2-yl]-piperidin-1-yl}-biphenyl-4-yl-methanone (16.a.4) (Figure 7.6), was found to be promising as it caused bacterial cell elongation and also inhibited the growth of many pathogenic bacteria including *S. aureus, S. pneumoniae, Haemophilus influenzae*, and *Moraxella catarrhalis*. In another study, a library of ~250 000 compounds was screened, using a fluorescence polarization assay to search for inhibitors of ZipA–FtsZ association. Twenty-nine hits that inhibited the binding of ZipA to FtsZ by at least 30% were identified [144]. One compound, pyridylpyrimidine (Figure 7.6), displayed profound efficiency toward binding to ZipA ($K_I = 12\,\mu M$) and was suggested to disrupt ZipA–FtsZ association by binding to the same hydrophobic patches where FtsZ was predicted to interact with ZipA [144].

Similarly, the membrane-anchoring protein, FtsA, can also be exploited to develop antibacterial agents. FtsA, a homolog of actin, has been reported to have ATP-binding property [49, 145]. Using a phage display approach, peptides were identified in *P. aeruginosa*, which target the ATPase activity of FtsA, although the significance of the ATPase activity is still uncertain [146]. Studies have shown that FtsA interacts with FtsZ through the C-terminal domain and the residues

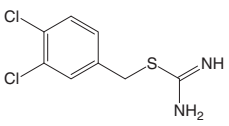

Figure 7.7 Structure of MreB inhibitor (A22) [150].

involved in the binding have been identified in *E. coli* [147–149]. Therefore, inhibitors perturbing the association of FtsA and FtsZ could be designed. Efforts are on to identify the mechanism of interaction of the early and late division proteins with FtsZ and among themselves, so as to develop inhibitors to effectively block the division process. Targeting these cell-division-related proteins may serve to identify compounds that have an adverse effect on bacterial cell division.

The proteins involved in various crucial functions in a dividing cell can also be considered putative targets for the development of antimicrobial agents. For example, an inhibitor targeting the cytoskeletal protein, MreB, has been identified recently. S-(3,4-dichlorbenzyl) isothiourea (A22) (Figure 7.7) was found to depolymerize MreB filaments in *E. coli* and *Caulobacter crescentus* cells. It induced the formation of spherical cells in *E. coli* [80] and also decreased the growth rate of *E. coli* cells at a concentration of 100 $\mu g\,ml^{-1}$.

7.4 Status of FtsZ-Targeting Compounds: From Laboratory to Clinic

During the past few years, many FtsZ-targeted agents have been reported from various laboratories across the world. At present, most of the compounds have been tested only under *in vitro* conditions. Some of these compounds have limitations, which might make them unsuitable as prospective drug candidates. Many inhibitors targeting FtsZ have been found to be effective in the micromolar range, which might decrease their therapeutic value as it becomes difficult to achieve such high concentrations when administered in patients. Optimization of the interaction between these molecules and FtsZ using structure–activity relation data could fetch molecules with substantial therapeutic values. However, some FtsZ-targeted agents display cytotoxicity in mammalian cells, as FtsZ and tubulin share structural similarities. Nevertheless, there are certain regions in the tubulin and FtsZ that are considerably different so that some of the FtsZ-targeted molecules do not show toxicity in mammalian cells. These differences in the structures of FtsZ and tubulin also provide sufficient scope for designing FtsZ-specific molecules using the structure-based drug designing approach.

In addition, inadequacy of animal model studies has left some of the promising compounds at the *in vitro* testing stage. This might be one of the prominent reasons that despite having so many FtsZ inhibitors, their clinical status remains ambiguous. Only PC190723 has been tested in murine models and has shown promising results against *S. aureus* infection in these animals [108]. *In vivo* model studies are followed by preclinical and clinical trials, which take several years.

Currently, none of the FtsZ-targeting molecules have been reported to be in clinical trial, which means there is a long wait before we see any of these molecules as a drug in clinics.

7.5 Conclusion

Targeting the cell division machinery of bacteria is an active area of research for the antibacterial drug developmental endeavor. Although modest success has been achieved in identifying small molecule inhibitors targeting FtsZ, very few cell division proteins have been targeted for the inhibition of bacterial cell division. Protein–protein interaction is clearly very important for the coordination of the cell division, with many proteins working simultaneously. A more elaborate understanding about the interaction of these proteins at both the structural and functional levels would facilitate the designing of inhibitory molecules or peptides. Further, the knowledge of their structures would help in specifically designing molecules that can perturb their individual functions. In addition to the common proteins, the cell division proteins, which are specific to certain pathogenic bacteria, can also be exploited for the development of more specific antibiotics. For example, WhiB2, PknA, PknB, and cell-wall hydrolases such as ChiZ [151–154] are cell division proteins that are restricted to the *Mycobacterium species*. Thus, the identification of the differences in the cell division machineries of different bacteria will provide new and more explicit targets.

It is apparent that targeting any of the key proteins of cell division may offer a good solution to arrest bacterial proliferation. The machinery is so huge that it provides many points of intervention, which can be exploited through more rigorous and focused research. With active research in the quest for the inhibitors of bacterial cell partitioning, it is possible that these inhibitors may emerge as effective tools to curb bacterial infections in the future.

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Abbreviations

GTP	Guanosine-5′-triphosphate
GDP	Guanosine-5′-diphosphate
PALM	Photoactivated localization microscopy
FRAP	Fluorescent recovery after photobleaching
K_d	Dissociation constant

FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
ITC	Isothermal titration calorimetry
IC_{50}	Half-maximal inhibitory concentration
MIC	Minimal inhibitory concentration
ED_{50}	Effective dose-50%
MRSA	Methicillin-resistant *Staphylococcus aureus*
MDRSA	Multidrug-resistant *Staphylococcus aureus*
NMR	Nuclear magnetic resonance
STD-NMR	Saturation transfer difference-nuclear magnetic resonance
2D HSQC–NMR	Two-dimensional heteronuclear single quantum coherence–nuclear magnetic resonance

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8
The Membrane as a Novel Target Site for Antibiotics to Kill Persisting Bacterial Pathogens

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8.1
Introduction

With the escalating burden of multiply drug-resistant pathogens and the diminished number of available antibiotics to treat bacterial infections, the bacterial cytoplasmic membrane has emerged as a bona fide target for developing novel agents with potent modes of action [1]. Traditionally, the cytoplasmic membrane was not considered a viable antibacterial target because of the potential for membrane-active agents to also disrupt the mammalian membrane, producing cytotoxic effects [2, 3]. Although this perception is quite valid, the clinical development of the membrane-active antibiotic daptomycin [4], and more recently telavancin [5], coupled with our increased understanding of the mode of action of antimicrobial defense peptides [6], has advanced the membrane as a natural and druggable target for antibiotics that can be selective for bacteria. A number of academic and industrial antibiotic discovery and development programs have therefore emerged, focusing on optimizing the antibacterial action of antimicrobial peptides, their nonpeptide mimetics or organic molecules that target the cytoplasmic membrane [1, 7, 8]. These programs represent an expansion of the traditional model of antibiotic discovery [9, 10] that primarily focused on inhibiting one of the five major biosynthetic processes in bacteria: that is, the biosynthesis of proteins, RNA, DNA, folic acid, and peptidoglycan [11]. Arising from these discovery programs are numerous reports demonstrating that membrane-active antibiotics display exceptional chemotherapeutic properties including rapid bactericidal action, activities against multidrug-resistant pathogens and low prospects for the emergence of resistance [1, 12]. Besides these attributes, an added advantage displayed by several membrane-active antibiotics is their ability to eradicate bacteria residing in physiologically dormant states (i.e., slow-growing or nongrowing subpopulations with lowered metabolic activities). This is an admirable property, considering that dormant bacteria are often refractory to killing by established classes of bactericidal antibiotics and the type of agents required to kill dormant bacteria was relatively unknown. Consequently, the finding that several membrane-active antibiotics kill persistent bacteria is an evolving paradigm for treating infections that persist. In this chapter, we examine this evolving

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concept, with a review of their modes of action and clinical and laboratory-derived examples of dormant cell killing by membrane-active agents. We also discuss some of the obvious limitations for the discovery and clinical development of such agents. Initially, we briefly describe why dormant bacteria are hard to eradicate, which illustrates why targeting the membrane is a novel and expanding paradigm for treating persistent infections. A comprehensive review of the membrane and associated bioenergetics as drug targets has also been described elsewhere [1].

8.2 The Challenge of Treating Dormant Infections

The acquisition of *de novo* mutations in target genes or mobile DNA containing antibiotic resistance genes are well-documented mechanisms by which bacteria grow in the presence of elevated concentrations of antibiotics and cause treatment failure [13, 14]. However, these are not the sole mechanisms, and may not even be the primary mechanisms [15–18], adopted by bacteria to subvert antibiotic-mediated killing. While many factors contribute to therapeutic failure, such as the suboptimal pharmacokinetics and pharmacodynamics of antibiotics and patient characteristics [19–21], it is now well accepted that infections that contain slow or nongrowing bacteria are hard to treat, unlike infections primarily associated with actively growing cells [9, 16, 22]. Clinical examples where antibiotic treatment failure coincides with the occurrence of dormant bacteria, include staphylococcal biofilms found in endocarditis and medical-device-related infections, cystic fibrosis caused by *Pseudomonas aeruginosa* [23, 24], recurrent streptococcal otitis media and sore throat [17, 25], ischemic osteomyelitis [26], nonhealing chronic wounds [27], and tuberculous granuloma caused by latent *Mycobacterium tuberculosis* [18]. Such infections are typified by the need for prolonged treatment periods before a clinical cure can be achieved; for example, the effective therapy of tuberculosis (TB) requires a 6-month regimen consisting of antibiotic combinations [18]; and a minimum of 4 weeks of treatment is needed for staphylococcal endocarditis and osteomyelitis [28, 29]. Unfortunately, for biofilm-mediated infections, antimicrobial treatment failure is common and requires the surgical removal of the infected devices or wound tissues colonized by biofilms [27, 30, 31]. The need for prolonged periods of treatment also increases the risk of patient noncompliance and the selection of genetic mechanisms of resistance [22].

During the development of an infection, bacteria first enter into a period of rapid growth, but this declines as the infection progresses because of nutrient limitations and the response of the immune system. The effect this has on antibiotic efficacy was appropriately described by Eagle in 1952 [32], showing that even high concentrations of penicillin failed to eradicate streptococci in the older stages of infection in mice. The poor efficacy of penicillin was attributed to bacteria occurring in a nonmultiplying state with low metabolic activity. However, the correlation between nongrowth, treatment failure, and the need for prolonged treatment is best typified by TB infections, where the actively growing populations are the first to

be eradicated by antitubercular therapy with the dormant subtypes accounting for the lengthy treatment period [33, 34]. Growth resumes when favorable conditions reoccur, resulting in actively growing cells that are more susceptible to killing by antibiotics [16]. In addition, in an infection, various subtypes of dormant bacteria are likely to coexist. Therefore, the antibiotic susceptibilities of cells within the dormant population are also likely to vary [1, 22], which implies that no single agent may effectively kill all subpopulations of dormant cells. The occurrence of mixed populations is evident in both TB and biofilm-mediated infections, where the bacterial community consists of cells exposed to acidic pH, and/or low nutrient availability and/or hypoxia [33, 35]. These conditions slow and eventually stop the growth of bacteria, resulting in dormant cells [16, 36].

So why do most of our antibiotics lack efficacy against dormant bacteria? This has much to do with the manner in which most agents were discovered as molecules that inhibit macromolecular biosynthesis and corrupt processes essential to logarithmically growing bacteria. The drawback of this approach is that dormant cells undergo a genetically regulated downshift in active cellular metabolism, causing a substantial decrease in the activity of several biosynthetic processes, including those that are targeted by many established antibiotics [9, 16, 22, 37, 38]. It is therefore presumed that the targets for most antimicrobial agents are either absent, or only occur in limited amounts, and, even if present, that the corruption of processes required for rapid growth are not deleterious to the survival of metabolically inactive bacteria [1, 15]. For example, β-lactam antibiotics kill by activating peptidoglycan hydrolases and this requires active peptidoglycan synthesis in growing cells. Also, tobramycin and ciprofloxacin preferentially kill metabolically active cells within the peripheral regions of *P. aeruginosa* biofilms [39]. Notable examples where some established agents do kill some subpopulations of dormant bacteria include rifampicin and moxifloxacin, which inhibit RNA and DNA synthesis, respectively, and display activity against some biofilms and *M. tuberculosis* [40–43].

8.3
Discovery Strategies to Prevent or Kill Dormant Bacteria

Given the metabolic differences between active and dormant cells, the targeting of proteins that are critical to the physiological adaptation and maintenance of dormant phenotypes have been regarded as cotherapy approaches for persistent infections [44]. PhoU, a protein that acts as a global repressor of cellular metabolism, is a leading example of the potential of this approach. The inactivation of PhoU in *Escherichia coli* or *M. tuberculosis* produces derivatives that are metabolically hyperactive and therefore easier to eradicate with antibiotics [45, 46]. Therefore, as an antidormancy target, the inhibition of PhoU may hinder the metabolic shift down for cells to become dormant [45]. However, from many studies it is apparent that bacteria utilize multiple genetic mechanisms to adapt to life in a nondividing state and a redundancy in functions exist [47–49]. This complicates

target selection for drug discovery processes. For example, the ribosome-associated proteins RelA and SpoT, which are responsible for the production of the global regulator (p)ppGpp, enable cells to respond to nutrient starvation as part of the stringent response [50]. As reported by Nguyen *et al.* [51], disruption of both RelA and SpoT was required to reduce the number of ofloxacin-tolerant cells by three log units in *P. aeruginosa* biofilms. Thus, both RelA and SpoT would need to be inhibited in some bacteria to abolish the role of the stringent response and cause cells to be more susceptible to antibiotics. Interestingly, ofloxacin failed to sterilize the biofilm of SpoT/RelA mutants [51], suggesting that additional mechanisms are also responsible for recalcitrance. A further complication is that dormant cell types may be present in an infection before the administration of therapy [9, 22] and strategies targeting their formation may be ineffective. Indeed, once bacteria attain a persistence state, they may diminish the role of enzymes needed for the initial transition stages. This view is exemplified by the signal transduction DosR/S system that regulates more than 50 genes and is central to *M. tuberculosis* adaption to hypoxia [52]. Accordingly, DosR initially emerged as an attractive antitubercular target [52], but findings of Rustad *et al.* [53] indicate that DosR is only important to the initial metabolic shift down in low-oxygen conditions and is not required for *M. tuberculosis* to endure hypoxia after adaptation has occurred. Another way forward to obtain agents that kill dormant bacteria would be to derail the cellular processes or targets that are essential to bacterial viability in both growing and nongrowing physiological states, as we articulate here for the membrane.

8.4
Why Targeting the Membrane Could Be a Suitable Strategy

In this section, we lay out why targeting the membrane is a suitable strategy for novel antibiotic discovery. We also describe the general mode of action of these agents. It is already evident from numerous research studies that the membrane is essential, selective killing of bacterial pathogens is achievable, and membrane-active agents typically exhibit a multitarget mode of action that results in potent bactericidal properties and low prospects for resistance emergence. For clarity, the membrane as a target site includes both the phospholipid bilayer and embedded proteins whose function may be inhibited by agents accumulated in the bilayer (Figure 8.1).

8.5
Target Essentiality and Selectivity

Irrespective of the cell's physiological status, the integrity of the membrane is essential because it provides a selective permeability barrier to ensure cellular homeostasis and metabolic energy transduction [1, 54]. The membrane also serves as the site for about a third of the cell's proteins, many of which perform critical processes including cell–cell communication in biofilms; active transport

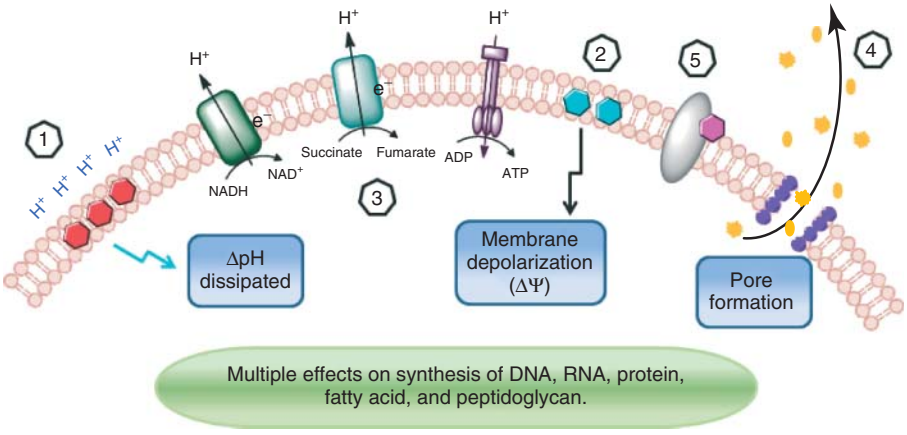

Figure 8.1 A generalized view on the action of agents targeting the bacterial membrane, indicating that the modes of action are complex and impose multitargeted effects. From membrane damage numerous cellular functions are affected, including macromolecular synthesis. (**1**) Some agents act as proton ionophores to dissipate the transmembrane pH gradient (ΔpH). (**2**) Others may act to specifically dissipate the membrane potential (ΔΨ); or both parameters are dissipated by some other agents. (**3**) It is likely that some agents will disrupt the electron transport chain (ETC) by either sterically binding to ETC. (**4**) Proteins acting as lipophilic redox molecules such as clofazimine. Other molecules affect the permeability of the membrane bilayer and cause pores to form, from which cytoplasmic contents are leaked from the cell and cells may rapidly die. (**5**) An often underexamined property in the mode of action of some compounds is the potential to specifically bind to proteins embedded in the bilayer. This binding may be part of the killing mechanism and also could engender specificity for prokaryotes if they lack mammalian counterparts. It should be noted that not all agents would exhibit these actions or even similar actions as this depends on drug structure and interactions made within the membrane.

of nutrients and wastes; bacterial respiration; ATP generation; and establishment of the proton motive force (PMF), sum of the transmembrane pH (ΔpH), and the membrane potential (ΔΨ) [55]. Consistent with its essential role, the bacterial membrane is the target for host-mediated defense antimicrobial peptides (AMPs) and several bioactive molecules found in both terrestrial and marine ecosystems [3, 56–58], which validates its significance as a natural antimicrobial target site. However, the development of membrane-active molecules has been hindered because of reasonable concern over the potential for cross-reactivity with the mammalian cytoplasmic membrane [3]. Nevertheless, the recent successful medical use of the membrane-active antibiotics daptomycin (a cyclic lipopeptide) and lipoglycopeptides (telavancin, oritavancin, and dalbavancin) against gram-positive infections indicate that bacterial specificity is therapeutically achievable. In addition, a wealth of literature exists on antimicrobial peptides and mimetics (peptide and nonpeptide) that specifically interact with the bacterial membrane [59–63]. The selectivity of these agents is due to their preferential interaction with the negatively charged phospholipids (i.e., acidic phospholipids, phosphatidylglycerol, and cardiolipin) that are

found in the outer leaflet of bacterial membranes. In contrast, mammalian membranes are characterized by an abundance of cholesterol (25%) and the presence of zwitterionic phospholipids; negatively charged phospholipids are rare in the outer leaflets of mammalian cells [64]. These differences allow daptomycin to selectively act on bacteria, where it first oligomerizes in the presence of calcium ions (Ca^{2+}) to form micelle-like amphipathic structures that are pseudopositively charged, with the hydrophobic decanoyl side chain facing inwards [65]. This pseudopositive structure increases daptomycin's affinity to the negatively charged membranes, as the Ca^{2+} is strongly drawn to the phosphatidylglycerol headgroups [65]. Interaction with the membrane then causes the micelle to dissociate, allowing the insertion of daptomycin's hydrophobic tail, in a manner analogous to the action of cationic antimicrobial peptides [65]. The selectivity of oritavancin also appears to be mediated by the positively charged molecule interacting with bacterial phosphatidylglycerol and cardiolipin, as shown by studies with model membranes [66, 67].

There is a need for studies to determine whether binding to the phospholipid bilayer is the sole determinant for selectivity or cell envelope (proteins and peptidoglycan) components are involved [63]. Indeed, the selectivity of some membrane-active molecules may be achieved by binding to peptidoglycan components and/or membrane-embedded proteins (Figure 8.1). For example, the cationic lantibiotic nisin disrupts bacterial membranes by first binding to the peptidoglycan precursor lipid II and undergoes a conformational change, allowing its hydrophobic moiety to insert into the bacterial membrane to form pores [68]. Nisin only disrupts the membrane of erythrocytes at concentrations 1000-fold above that required for antimicrobial activity, indicating its selectivity for bacteria [69] and presumably the role of binding to lipid II, which is absent in mammalian cells. Binding to cell-wall components also contributes to the specificity of lipoglycopeptides [12] and potentially daptomycin [70]. Interaction with cell-wall components is therefore a property worth considering to optimize underexplored membrane-active chemotypes to specifically interact with bacterial cells.

8.6 Multiple Modes of Actions

Without an intact membrane, cells are no longer viable. From this, it is clear why detergents that are lytic and cause physical damage to the membrane are bactericidal. However, several membrane-active agents with potent antibacterial action are nonlytic, indicating that this class of agents displays a complex mode of action not likely restricted to physical membrane damage [65, 67, 71]. In general, the mode of action of membrane-active agents may involve the interaction of hydrophobic chemical groups of the compounds with the bacterial membrane; the aggregation of molecules within the membrane, which disrupts its structure and functional integrity and may lead to leakage of cytosolic contents; steric inhibition of membrane-embedded proteins; and alteration of the PMF and bacterial redox, which may cause eventual cell death (Figure 8.1) [1]. These actions often result in the

simultaneous inhibition of several macromolecular processes that are individually inhibited by specific classes of antibiotics. However, the modes of action for different membrane-active molecules may be very dissimilar even between structural-related classes because not all agents will interact with the membrane in similar ways. Rather, these effects may depend on the structure of the antimicrobial agent and the sum of its interaction within an environment as complex as the bacterial membrane. This is shown by cylic lipopeptide antibiotics that interact with the cell envelope (membrane and cell wall). For example, high concentrations of the lipopeptide MX-2401 (i.e., 32 $\mu g\,ml^{-1}$ or 16 $\times$ its MIC) is required to depolarize the membrane of *Staphylococcus epidermidis*, whereas daptomycin causes membrane depolarization at only 1 $\mu g\,ml^{-1}$ (i.e., 2 $\times$ MIC) [72]. The mode of action of MX-2401 is explained by its binding to the bactoprenolphosphate (C_{55}-P) carrier, thereby inhibiting peptidoglycan biosynthesis, while daptomycin does not appear to affect C_{55}-P reactions [72, 73]. Perhaps the greater rigidity of MX-2401 compared to daptomycin in the presence of Ca^{2+} may account for the differences in the mode of action of these related compounds [72].

While it is evident that detergent-like pore formation and disruption of the membrane structure would be bactericidal to even dormant cells, the dissipation of the membrane potential alone is not necessarily bactericidal in all species, although this property could possibly limit the supply of energy in metabolically inactive cells. Therefore, the identification of molecules that affect the membrane potential does not necessarily imply they will have activity against biofilms, stationary phase cells, or other dormant cell types. For example, dissipation of the membrane potential ($\Delta\Psi$) and transmembrane pH gradient (ΔpH) by valinomycin and nigericin, respectively, only imposes a bacteriostatic effect on most bacteria such as *Staphylococcus aureus* [74–76]. In contrast, it is striking that the disruption of these parameters induces cell death in actively growing and dormant *M. tuberculosis* [77] and *Clostridium difficile* (see subsequent text) [78]. This signifies that a fully energized membrane may be critical to *M. tuberculosis* and *C. difficile* unlike other pathogens. Hence, agents that dissipate the bacterial PMF, in addition to imposing steric multitarget effects, are likely to be highly effective in sterilizing both growing and nongrowing *M. tuberculosis* and *C. difficile*. Furthermore, by increasing membrane proton permeability, mycobacteria would become more sensitive to killing by reactive free radicals (nitric oxide and superoxide) in macrophages, as this mechanism is enhanced at acidic pH [79].

8.6.1
Bactericidal and Low Potential for Resistance Development

For life-threatening infections characterized by a high bacterial load in tissues and the poor response of bacteria to antibiotic therapy, the use of bactericidal agents with a limited potential for resistance development is desirable. Examples of such infections where bactericidal activity is favored include pulmonary infections in immunodeficient patients and infections where bacteria are protected from the immune response, such as within a biofilm matrix and tuberculous granuloma

[80–82]. An added benefit of membrane-active agents is the limited resistance potential of these agents, which may be lower than that of many established antibacterial drugs. This property is favorable for their therapeutic use as the rate with which bacterial resistance emerges following clinical introduction of antibiotics dictates the length of the drug's useful life span [1]. Lower rates of resistance development has been observed with several membrane-targeting compounds including daptomycin [147], telavancin [83], reutericyclins [84], porphyrins [85], a variety of cationic antimicrobial peptides [13] and HT-61, a quinolone-derived membrane-active compound [10]. This suggests that *de novo* mutations causing resistance to membrane-active antibiotics do not readily arise or fitness costs preclude the maintenance of resistant mutants. According to Chopra and colleagues [86], if the action of membrane-active compounds were due to insertion into the membrane bilayer, then changes in membrane composition would be required to alter the physicochemical interactions. In such cases, multiple mutations that may be incompatible with the survival of the cells could be required. However, not all molecules that target the membrane will have a low potential for resistance, as this property seems to be guided by the structure of molecules and the extent of their interactions within the cell envelope (i.e., interaction with the membrane bilayer, embedded proteins, and cell wall). For example, nisin-resistant *S. aureus* arises at a high frequency of 10^{-7}, comparable to fusidic acid and rifampicin, and is due to there being multiple routes for nisin resistance to emerge [86]. Resistance arising from the expression of efflux pumps may also cause subversion of killing. This is evident in *P. aeruginosa* that responds to cationic peptides by induction of the lipopolysaccharide modification system (e.g., *pmrAB* operon) and expression of efflux pumps (e.g., *mexAB-oprM*) [87, 88]. However, the rapid elimination of pathogens that are inherently susceptible to membrane-active drugs should reduce the likelihood of stepwise resistance emergence, provided concentrations remain bactericidal at the site of infection.

8.7 Therapeutic Use of Membrane-Damaging Agents against Biofilms

Biofilm-mediated infections are particularly difficult to treat owing to fact that cells within the biofilm are physiologically heterogeneous [35]. The biofilm population consist of cells that are metabolically stratified, that is, slow growing or nongrowing bacteria; cells that are exposed to oxygen, microaerophilic, or anaerobic niches; cells that are either supplied with nutrients or nutrient deprived; and those that are exposed to fermentation-derived acids that can inhibit growth [35, 89]. Therefore, in order for a single agent to be effective against biofilm-mediated infections it has to retain activity against multiple cell types, that is, being able to corrupt cellular processes that are essential to all cells in the population. As mentioned, the bacterial membrane function is critical to all viable cells irrespective of their metabolic status.

A number of agents targeting the bacterial cytoplasmic membrane are known to possess activity against biofilms and nongrowing cells *in vitro* (Table 8.1). Among these molecules, the antibiotic daptomycin was introduced into the clinic

Table 8.1 Antibacterials with action against various dormant bacteria: mode of action and development status.

Antibiotic	Microbiology	Mode of action	Antibiotic status	References
Daptomycin	Gram-positive bacteria (e.g., *S. aureus* and *Enterococcus* spp.); active against biofilms	Membrane permeabilization and depolarization; disrupts multiple cellular processes	Approved (2003) for cSSSI; *S. aureus* bacteremia and right-side endocarditis	[100]
Telavancin	Gram-positive bacteria (e.g., *S. aureus* including VISA); active against biofilms	Inhibits peptidoglycan biosynthesis by binding to the D-Ala-D-Ala termini; membrane permeabilization and depolarization; disrupts multiple cellular processes	Approved (2009) for cSSSI; completed Phase III for pneumonia	[5]
Oritavancin	Gram-positive bacteria (*S. aureus* including VISA and *Enterococcus* spp.); active against biofilms and stationary phase cells	Inhibits peptidoglycan biosynthesis by binding to the D-Ala-D-Ala termini; membrane permeabilization and depolarization; disrupts multiple cellular processes	Completed phase III for cSSSI	[5]
Dalbavancin	Gram-positive bacteria (e.g., *S. aureus*, including VISA); shown to prevent biofilm formation *in vivo*	Inhibits peptidoglycan biosynthesis by binding to the D-Ala-D-Ala termini; alternate modes of action presumed to involve membrane disruption	Completed phase III for cSSSI	[5, 114]

(*continued overleaf*)

Table 8.1 (*Continued*)

Antibiotic	Microbiology	Mode of action	Antibiotic status	References
Reutericyclin (Lee-867)	Gram-positive bacteria (e.g., *S. aureus*); active against biofilms	Membrane depolarization; disrupts multiple cellular processes	Discovery stage for topical use against *S. aureus* and *C. difficile*	[84]
XF-73	Gram-positive bacteria (e.g., *S. aureus*)	Membrane permeabilization and depolarization; disrupts multiple cellular processes	In clinical trials for nasal decolonization of *S. aureus*	[99]
CSA-13	Broad spectrum; active against biofilms	Membrane permeabilization and depolarization; disrupts multiple cellular processes	Preclinical stage for topical use and biofilm prevention on medical devices	[105, 119]
HT-61	Gram-positive bacteria (e.g., *S. aureus*)	Membrane permeabilization and depolarization; disrupts multiple cellular processes	In clinical trials for nasal decolonization of *S. aureus*	[106]
LTX-109; LTX-5	Broad spectrum; active against biofilms	Membrane permeabilization and depolarization; disrupts multiple cellular processes	LTX-109 completed phase II clinical trials for nasal decolonization of *S. aureus*	[108]
Clofazimine derivatives	*M. tuberculosis* and *S. aureus;* active against nondividing cells	Complex action, including membrane depolarization	Discovery optimization stage	TB Alliance[a], [182]

VISA, vancomycin-intermediate *S. aureus*; cSSSI, complicated skin and skin structure infections.
[a]TB Alliance (The Global Alliance for TB Drug Development *www.tballiance.org/*).
Source: Modified from Hurdle *et al.* [1].

in 2003 and is now established for the treatment of staphylococcal and enterococcal infections. More recently, telavancin was approved for clinical use in North America (in 2009) and Europe (in 2011) for complicated skin and skin structure infections caused by gram-positive bacteria, including *S. aureus* [90]. Studies show that daptomycin displays a more rapid kill of staphylococcal biofilms than the majority of other established antibacterials, in some cases demonstrating complete biofilm eradication [91–93]. In a murine model of foreign-body infection with two different strains of *S. epidermidis*, daptomycin improved survival by 66.7 and 93.3% compared to vancomycin 42.9 and 76.9% [93]. Corroborating these studies are clinical reports that daptomycin is effective against both staphylococcal and enterococcal endocarditis [93]. However, daptomycin may not prove useful in eradicating all types of staphylococcal biofilm infections as its use against catheter-associated biofilms in mice over a period of 7 days only led to clearance of the biofilm in less than 7% of cases [94]. The lack of daptomycin efficacy against some biofilms does not appear to result from poor diffusion through the polysaccharide matrix as daptomycin rapidly penetrates into biofilms [96]. Rather, efficacy may in part be limited by physiologically available levels of calcium as shown by John *et al*. [97], where increases in calcium concentrations led to improved efficacy in a foreign-body infection in mice. Nevertheless, combination therapies may be needed to sterilize certain biofilm diseases, as the clinical efficacy of daptomycin, as in all other drugs, will depend on the pharmacokinetics and pharmacodynamics of the drug at the site of infection. In addition, daptomycin requires higher concentrations (an increase from 2 to 32 μg/mL) to achieve killing of stationary phase bacteria, in contrast to logarithmic cells, exemplifying that not all membrane-damaging agents will be effective against certain dormant infections [98]. These findings on daptomycin do not imply that other structurally distinct membrane-targeting molecules will lose bactericidal activities against the different forms of slow growing or dormant bacteria, as is seen for lipoglycopeptides and XF-73 [99, 110].

Telavancin is reported to eradicate staphylococcal and enterococcal biofilms at concentrations close to those required to kill planktonic counterparts [111]. The minimum biofilm eradication concentration (MBEC) of telavancin against *S. aureus, S. epidermidis*, and *Enterococcus faecalis* ranged from 0.12 to 2 $\mu g\,ml^{-1}$ and was comparable to the minimum bactericidal concentrations of 0.12–1 $\mu g\,ml^{-1}$ against planktonic cells. Similarly, the related lipoglycopeptide oritavancin is highly effective in killing staphylococcal biofilms at low concentrations (0.5–8 $\mu g\,ml^{-1}$) that are within a doubling dilution of the MICs [110]. Although telavancin and oritavancin inhibit peptidoglycan biosynthesis, this mode of action is unlikely to account for their antibiofilm properties, as the prototypical vancomycin fails to kill nongrowing cells including biofilms (MBEC $> 512\,\mu g\,ml^{-1}$). Instead, the killing of dormant cells by lipoglycopeptides appears to correlate with their hydrophobic side chains permeabilizing the bacterial membrane to cause multiple cellular effects (Figure 8.1) [110–113]. The incorporated hydrophobic moieties confer strong dimerization properties on telavancin and oritavancin, allowing for enhanced interaction with the membrane and increased affinities for peptidoglycan precursors [111–113]. Another lipoglycopeptide, dalbavancin, derived from teicoplanin,

contains a fatty acyl and C-terminal dimethyl-aminopropyl groups that are believed to also enable its dimerization and insertion into the bacterial membrane [113]. Hence, dalbavancin may be active against dormant cells, but detailed studies on its mode of action against such cell types are presently lacking. Nonetheless, Darouiche *et al*. [114] reported that dalbavancin prevented *S. aureus* colonization of catheters in rabbits, with some improvement over vancomycin. As clinical and laboratory studies continue for these membrane-active lipoglycopetides, it is plausible that they could emerge as treatments for persistent osteomyelitis, endocarditis, and catheter-related infections [90, 115].

A number of other membrane-active molecules at various stages of clinical, preclinical, or experimental development also display potent activity against biofilms and dormant cell types (Table 8.1, Figure 8.2). An interesting class of molecules is the novel porphyrin antistaphylococcal agents, for example, XF-73, being developed by Destiny Pharma. Within 10 min of exposure, XF-73 causes rapid depolarization of the staphylococcal membrane with depletion of ATP and abolishment of DNA, RNA, and protein synthesis, but without detergent-like cell lysis [85]. Importantly, XF-73 achieves eradication of *S. aureus* biofilms and stationary phase cells at concentrations close to the MIC (1 μg ml^{-1}) for planktonic cultures [99]. Also, derivatives of the tetramic acid natural product reutericyclin (Figure 8.2), which are efficacious in murine skin infections and disrupt the bacterial membrane potential, kill staphylococcal biofilms (e.g., Lee-867 shows MBECs of 6.25–50 μg ml^{-1}) [85]. Several antimicrobial peptides also display biofilm killing, but a noted disadvantage is the susceptibility of some peptides to proteases and electrostatic repulsion or retention by the charged exopolysaccharide matrix [107, 116]. Mimetics of antimicrobial peptides provide an alternative. An example is LTX-109 (from Lytix Biopharma AS) that is in clinical trials for nasal decolonization of MRSA (methicillin-resistant *S. aureus*) and treatment of gram-positive skin infections. The earlier generation of molecules related to LTX-109 (e.g., LTX-5) was shown to eliminate metabolic activity and sterilize biofilms of *S. epidermidis* and *S. hemolyticus* at $\leq 10 \times$ their MICs (4–8 μg ml^{-1}) [117].

The adherence of biofilms to the surface of medical devices provides a route for bacteria to disseminate through the body and cause serious systemic infections [17, 118]. For example, the contamination of venous catheters by *S. aureus* and *S. epidermidis* on the skin often leads to staphylococcal bacteraemia [118]. The immobilization of antibiotics to medical device surfaces (''smart surfaces'') is one way to prevent their contamination by biofilm-forming bacteria such as staphylococci. Because membrane-active agents do not rely on intracellular targets for their activities, these molecules may be advantageous over other antibiotics that have intracellular targets. This is demonstrated by chitosan and ceragenins; when coated on a device surface, these molecules prevented biofilm contamination and were substantially more effective than chlorhexidine. Chitosan was also shown to be more effective than minocycline and rifampicin [103]. Similarly, antimicrobial peptides [119, 120] such as melime [121, 122] have been covalently attached to devices in order to prevent bacterial contamination. Melime, when attached to contact lenses, reduced the incidence of infection and tissue damage by *S. aureus*

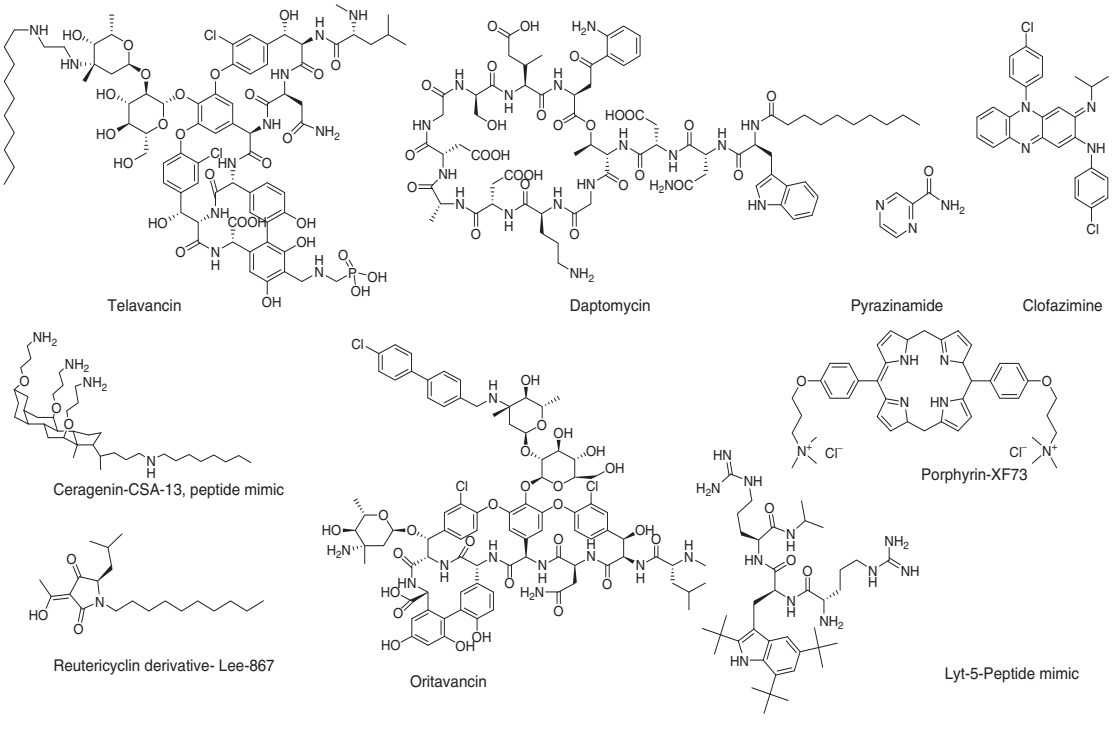

Figure 8.2 Chemical structures of different membrane-active agents. Molecules that perturb the membrane vary greatly in size and chemical structure; this influences the sum of the interactions made within the complex membrane environment, which may define how rapidly these compounds act, and whether or not they are bactericidal. A common feature of all membrane-active agents is their high lipophilic content that enables interaction with the hydrophobic membrane. With the exception of clofazimine and pyrazinamide, which kills latent mycobacteria, the antibiofilm properties of the compounds are reported. *Source*: Modified from Hurdle *et al*. [1].

and *P. aeruginosa* in animal models of ocular infections [121]. Although the mode of action of membrane-active agents is compatible with the ''smart surface'' approach, it may only apply to compounds that are not highly lipophilic and prone to becoming bound by serum proteins. Binding to serum proteins is known to reduce the efficacy and antimicrobial activity of highly lipophilic membrane-active molecules [1].

8.8
New Approaches to Identifying Compounds That Kill Dormant Bacteria

The classical route for discovering novel antibiotics involves identifying molecules that prevent the logarithmic growth of bacteria, but many of the agents discovered in this manner are either inactive or only partly active against dormant bacteria [9]. In order to identify novel molecules that kill dormant bacteria, Coates [9, 10, 22] have long advocated the need for discovery approaches focusing on dormant bacteria. In this regard, Coates and colleagues [10] performed a virtual screen of commercial libraries using quinolone as the pharmacophore, as fluoroquinolones such as moxifloxacin and gatifloxacin reduce the viability of persistent *M. tuberculosis* [40, 41]. Testing of the procured compounds against late stationary phase *S. aureus* identified two chemically related hit molecules that reduced the cell population by two to three logs. Subsequent medicinal chemistry efforts led to the discovery of the quinolone-like analog HT-61 that preferentially killed nongrowing staphylococci and other gram-positive organisms. The rapid bactericidal mechanism of HT-61 was reported to result from physical disruption of the cytoplasmic membrane, associated depolarization, and nicking of the cell wall. These mechanisms of action are not typical of fluoroquinolones that primarily prevent DNA replication by inhibition of DNA topoisomerases. Quinolone-like compounds have been reported to interact with components of malaria and toxoplasma respiratory chain, including nicotinamide adenine dinucleotide (NADH) type II dehydrogenase that is absent in mammalian mitochondria [123, 124]. Quinolone signal molecules (e.g., 2-heptyl-3-hydroxy-4(1*H*)-quinolone; PQS) from *P. aeruginosa* also act as respiratory inhibitors of *S. aureus* and kill this organism in biofilms [125, 126]. Therefore, quinolone-like HT-61 may potentially show simultaneous inhibition of bacterial respiration and the membrane bilayer. HT-61 is in phase III clinical trials for nasal decolonization of *S. aureus* and may be superior to the leading treatment mupirocin, particularly in patients who are persistent carriers.

8.9
Challenges for Biofilm Control with Membrane-Active Agents

While the above-mentioned examples are encouraging, they also reveal some challenges of current antibiofilm agents, their evaluation, and requirements to expand this area.

8.9.1 Test Methods

First, standardized methodologies for evaluating antibiofilm activity are lacking, with substantial variation in experimental approaches employed in different laboratories [9]. Some of the existing models include static biofilm devices (e.g., the modified Calgary biofilm device and colorimetric microtiter systems), where biofilms are grown under batch culture conditions, in contrast to flow biofilm models such as the Centers for Disease Control (CDC) bioreactor and BioFlux systems where biofilms are cultured with a continuous supply of fresh nutrients [127–129]. Hence, the activities of agents from different studies cannot be objectively compared. This is further complicated by the lack of standardized panels of test organisms and media conditions to examine compounds.

8.9.2 Spectrum of Activity

Most antibiofilm approaches do not take into account the fact that medical biofilms often have a polymicrobial composition of gram-positive and gram-negative organisms, such as in diabetic wound infections, cystic fibrosis, dental caries, and otitis media [130–133]. This certainly impacts the outcome of antimicrobial treatment. Therefore, an effective antibiofilm strategy will need to demonstrate coverage for multiple bacterial species or at least synergize with other antibiotics to achieve complete sterilization [15]. Owing to the presence of an outer membrane and multidrug efflux pumps, finding anti-gram-negative agents is proving to be an immense challenge [134]. There is therefore a real need to expand the use of membrane-active antimicrobials to gram-negative organisms and their biofilms. For this approach to be effective, such agents will either damage the outer membrane, as in the case of the drug colistin that has antibiofilm properties [87], transverse the outer membrane layer to reach the cytoplasmic membrane target and thereby cause damage to cells, or be suitable for combination drug use with antibiotics that permeabilize the outer membrane. In an interesting illustration of the latter opinion, Hornsey *et al*. [104] described colistin's potentiation of telavancin's activity against various gram-negative species, where the MICs of telavancin were reduced from > 32 to $\leq 1\,\mu g\,ml^{-1}$ in the presence of 0.25–0.75 μg of colistin ml^{-1}. The effect of this combination was not examined against biofilms, but, presumably, colistin-exposed cells have weakened outer membranes, allowing telavancin access to their cytoplasmic membrane and peptidoglycan precursors. This would enhance overall biofilm killing, seeing that colistin is less effective against the more metabolically active cells in the biofilm [87]. The antimicrobial peptide mimic CSA-13 belonging to ceragenins, a class of cationic steroid antimicrobials, exhibits broad-spectrum activity from its ability to permeabilize the outer membrane and cause cytoplasmic membrane depolarization [105, 135]. Ceragenins may represent a novel class of agents

that potentially controls polymicrobial infections because of their bactericidal activities against biofilms formed by different species including *S. aureus, P. aeruginosa*, and *E. faecalis* [137].

Although selectivity for bacteria is demonstrated for several membrane-active agents, there is still an abundance of compounds in chemical screening libraries that show antibiotic activity but also cause nonspecific lysis of mammalian membranes [3, 138]. The optimization of these hits to engender specificity presents a problem because little is known about the structure–activity relationships required for small membrane-active molecules to display specificity for prokaryotic membranes [15, 139]. It is plausible that this knowledge gap can be filled for some classes of molecules by applying concepts from antimicrobial peptides or peptidomimetics to systematically alter the hydrophobicity, charge, and amphipathicity of molecules [59, 140].

8.9.3 Pharmacological

From a medicinal chemistry perspective, membrane-active agents present problems for the design and development of derivatives lacking commonly associated toxicities (e.g., nephrotoxicity) and factors that affect drug disposition and efficacy (e.g., serum binding and tissue penetration) [1]. Most membrane-active drugs will tend to be partly lipophilic in nature for their interaction with the cytoplasmic membrane. Therefore, the systemic circulation of these drugs is hindered by the aqueous environment of the blood that is thermodynamically unfavorable for the disposition of hydrophobic drugs, which tend to be poorly soluble, extensively bound to plasma proteins, and not well distributed in all tissues [141]. This affects the distribution of drugs throughout the body, including into infected tissues. Nevertheless, these properties should not prevent the development of membrane-active agents, especially in light of the paucity of antimicrobials with novel modes of action encompassing dormant cells. Furthermore, their rapid bactericidal action should be advantageous in treating serious infections, where the bacterial load is high. Evidence that pharmacological challenges can be overcome is demonstrated by the development of daptomycin, where the key to its clinical introduction was the choice of dosing regimen. Patients are dosed daily with high concentrations of daptomycin with a long interval period, to maximize daptomycin's concentration-dependent killing of bacteria and to avoid myotoxicity that may occur with frequent doses of daptomycin [1, 94, 142]. There is also variation in the pharmacological properties of different compound classes. For instance, daptomycin is not useful for respiratory infections as it is bound to proteins in the bronchoalveolar lavage epithelial lining fluid (BAL-ELF) [142, 143], but telavancin, in spite of being highly protein bound (90–93%), compared to vancomycin (10–55%), attains unbound concentrations that are above its MIC against MRSA [5, 144].

The use of specialized formulations may also be required for some hydrophobic membrane-active agents to advance clinically. For example, the concentration of the unbound fraction and the toxicity of Amphotericin B that is used to treat systemic

fungal infections are significantly increased and decreased, respectively, when this antibiotic is delivered via liposomal formulations [145].

8.9.4 Genetic Resistance

Even though laboratory studies show that there is a low potential for resistance to arise to membrane-active antibiotics, resistance may eventually occur following widespread clinical use. This would certainly nullify the ability to treat both persistent and acute infections. For example, initial laboratory studies with daptomycin suggested a low rate of occurrence of daptomycin-resistant spontaneous mutants [146, 147]; but resistance to daptomycin was detected shortly after its clinical introduction [148, 149]. Presently, the prevalence of daptomycin-resistant strains in the clinic is exceedingly rare [150, 151]. The current understanding of daptomycin resistance in *S. aureus* indicates that it is multifactorial and partially mediated by overproduction of the enzyme lysylphosphatidylglycerol (LPG) synthetase (encoded by *mprF*) [152, 153] or increased flipping of the positively charged LPG to the outer leaflet [154]. The role of LPG synthetase in daptomycin resistance is supported by an increase in daptomycin susceptibility in strains with a deleted *mprF* or those expressing an antisense RNA to *mprF* [155, 156]. LPG synthetase has two domains: the LPG synthetase domain is responsible for producing LPG by addition of lysine to anionic phosphatidylglycerol and the flippase domain translocates LPG to the outer membrane leaflet. The activity of the flippase domain increases the net positive charge of the bacterial surface, which diminishes the binding of daptomycin to the membrane [153, 154, 157]. This mechanism also confers cross-resistance to cationic peptides (LL-37, protegrins, and nisin) [157], indicating it may be involved in protecting bacteria from the mammalian immune system [102]. However, not all compounds targeting the membrane will be affected by MprF because it appears that telavancin [83] and XF-73 [158] retain activities against daptomycin-resistant *S. aureus* with mutations in MprF. Expression of the *dltABCD* operon, causing the formation of alanyl-phosphatidylglycerol, also confers reduced susceptibility to daptomycin and cationic peptides due to an increase in net positive charge of the bacterial surface [154, 157].

The utility of some lipoglycopeptides may be affected by preexisting *vanA*-mediated resistance to vancomycin.The *vanA* gene cluster causes the production of peptidoglycan precursors ending in D-Alanyl-D-Lactate instead of D-Alanyl-D-Alanyl, which reduce the binding of glycopeptides to peptidoglycan by up to 1000-fold [159]. As a result, the MICs of dalbavancin ($> 32\,\mu g\,ml^{-1}$) and telavancin (MICs $= 2–16\,\mu g\,ml^{-1}$) are decreased against vancomycin-resistant *S. aureus* (VRSA) and vancomycin-resistant enterococci (VRE) expressing *vanA*, when compared to wild-type strains (MICs $\leq 4\,\mu g\,ml^{-1}$) [5, 83]. In contrast, oritavancin is active against clinical isolates of VRE and VRSA, as its well-dimerized form strongly interacts with the cytoplasmic membrane and binds to both D-Ala and D-lactate precursors [160, 161]. Overall, the clinical prevalence of *vanA*-mediated resistance in *S. aureus* is low, possibly due to fitness costs for the maintenance

of the *vanA* cluster [159, 162]. This could imply the long-term and widespread use of lipoglycopeptides to treat staphylococcal infections. As the numbers of membrane-active agents with clinical prospects expand, it will become increasingly important to evaluate their genetic mechanisms of resistance, using techniques such as genome sequencing and transcriptional responses to identify genes involved [163, 164] and the potential for cross-resistance among differing classes of agents.

8.10 Potential for Membrane-Damaging Agents in TB Disease

The World Health Organization (WHO) estimates that in 2009, there were 9.4 million new TB cases and 1.7 million deaths [165]. It is estimated that one-third of the world (2.15 billion) is asymptomatically infected with latent TB bacilli, which represent a large reservoir for new cases of active TB [18]. The metabolic inactivity of TB within the lung granuloma is the foremost reason why a 6–9 month regimen is required to achieve sterilization. This lengthy treatment period leads to therapeutic failure, noncompliance, and the emergence of drug resistance. Thus, a major goal of many antitubercular discovery programs is to develop drugs that shorten the duration of TB treatment to less than 2 months. In this regard, it is quite possible that membrane-active agents could shorten the duration of treatment for TB disease, as could be inferred from the potent antitubercular activities of energy uncouplers [1, 77, 166].

In an elegant series of experiments, Rao *et al*. [77] revealed that the PMF is required to maintain the viability of both active and nondividing *M. tuberculosis*. The ionophores nigericin and valinomycin specifically dissipate the transmembrane pH gradient (ΔpH) and membrane potential ($\Delta\Psi$), respectively. Using these two ionophores, Rao *et al*. [77] found that nongrowing *M. tuberculosis* was killed by concentrations that were bactericidal to growing cells. For nigericin, the bactericidal concentrations were 0.4 and 1.25 μM versus nongrowing and growing TB bacilli, respectively. Likewise, valinomycin was bactericidal at 0.5 and 1 μM, respectively, in contrast to isoniazid that did not kill dormant cells at test concentrations of 500 μM. Hence, both parameters of the PMF (i.e., $\Delta\Psi$ and ΔpH) are critical for the ATP production and survival of *M. tuberculosis*, indicating that these cells require a fully energized membrane [77]. It also explains why the inclusion of the drug pyrazinamide (PZA) as part of the TB regimen reduced the treatment period from 9 to 6 months.

PZA only kills metabolically dormant cells at acidic pH and under oxygen-limiting conditions, within acidified macrophages in the granuloma, by collapsing the PMF and depleting ATP, likely through dissipation of ΔpH [166, 167]. Despite its *in vivo* sterilizing activity, PZA is poorly active *in vitro* (MIC $\approx 60\,\mathrm{mg\,l^{-1}}$), even under acid conditions. It can therefore be hypothesized that by reducing the intracellular pH, PZA synergizes with host-derived reactive oxygen and nitrogen species [79] to achieve *in vivo* sterilization. Alternative modes of action may exist

for pyrazinamide, including inhibition of fatty acid synthase I (FAS I) biosynthesis [168] and prevention of trans-translation, which is instrumental for freeing scarce ribosomes in dormant cells [169]; the idea of FAS I being a target for pyrazinamide has been disproved by Boshoff *et al*. [170]. It may be that collapsing the ΔpH and inhibiting trans-translation are the reasons for the PZA sterilizing action. Nevertheless, inhibitors of energy metabolism such as the drug candidate TMC207 that inhibits mycobacterial ATP synthase and the antipsychotic thioridazine that targets mycobacterial type II NADH dehydrogenase (an electron transport chain, ETC enzyme) further validate the feasibility of targeting of *M. tuberculosis* energy production to shorten the duration of TB treatment [171–174]. Clinical trials with TMC207 and the off-license use of thioridazine indicate that both molecules are effective in treating patients with pulmonary TB [171, 173]. The development of antitubercular compounds targeting energy metabolism is now an established antitubercular approach, as shown for the ongoing development of agents: Ro 48–8071 that disrupts the ETC by inhibiting menaquinone biosynthesis A [175, 176]; or nitroaromatics such as the nitroimidazoles (PA-824 and OPC-67683) that are in current clinical trials [177], nitazoxanide [178], and nitrofurans (early discovery stage) [179]. The activities of nitroaromatics are associated with multitarget effects owing to their production of reaction nitrogen intermediates, NO poisoning of cytochrome oxidases [165], and dissipation of the PMF [180].

On the basis of these findings, membrane-active agents that dissipate the $\Delta\Psi$, ΔpH, or both, and cause ATP depletion are likely to cause cell death in *M. tuberculosis*. However, the concept of targeting the membrane to discover novel antimicrobials has not been widely applied to TB disease, with a dearth of examples in the literature. Indeed, the uncoupling of ATP synthesis and lowering of the PMF are common characteristics of membrane-targeting agents (Figure 8.1). The third-line drug clofazimine, a highly lipophilic-redox-active riminophenazine, whose mode of action is not well defined, provides one example. Studies have shown that clofazimine may affect the membrane architecture [181, 182], causing the accumulation of lysophospholipids and depletion of potassium and ATP that would correlate with membrane depolarization [109, 183]. It also appears that clofazimine, once accumulated in the membrane bilayer, undergoes an enzymatic reduction by mycobacterial type II NADH Dehydrogenase and its reduced form transfers electrons to oxygen to cause the formation of reactive oxygen species [183]. Consequently, clofazimine is bactericidal to dormant *M. tuberculosis* under hypoxia [184], shows near sterilizing activity in mice [185], and is effective against multidrug-resistant TB [182]. The side effect of unwelcome skin discoloration and variable pharmacokinetics due to its lipophilic properties may have limited clofazimine's widespread antitubercular use. However, less lipophilic, soluble derivatives of this molecule that retain activity against nondividing *M. tuberculosis* have been reported and could improve on the poor pharmacokinetics of clofazimine for treating TB disease [186].

Host-derived antimicrobial peptides are also active against *M. tuberculosis* [187], but their effects on dormant cells are largely unknown, although it appears that ubiquitin-derived peptides kill persister cells that are tolerant to

rifampicin (Georgiana E. Purdy, Oregon Health Sciences University, personal communication) [1]. Membrane-active peptidomimetics are also potential molecules being pursued for the treatment of TB disease, such as Oligo-*N*-substituted glycines [188] and defensin mimetics being undertaken by Polymedix (*http://www.polymedix.com/company-profile*).

8.11 Application to Treatment of *Clostridium difficile* Infection

The turn of the century saw a rapid increase in the number of cases of hospital-associated diarrhea caused by *C. difficile* in developed countries, with elderly patients being the most susceptible population [189, 190]. For example, in Quebec, Canada, the number of cases of CDIs (*Clostridium difficile* infections) increased from 35.6 per 100 000 persons in 1991 to 156.3 per 100 000 persons in 2003. In the United States, *C. difficile* causes an estimated 500 000 infections, with mortality in 15 000–20 000 cases per annum [189, 191]. This trend coincided with the emergence of hypervirulent strains designated as BI/NAP1/027 that produces copious amounts of the lethal toxins A and B in the late logarithmic (slow growing) and stationary phases of growth. Therefore, it seems plausible that by killing stationary phase cells the overall producing toxin population would be diminished, thereby causing a faster resolution of CDI. In addition, as sporulation normally occurs in late logarithmic and stationary phases, it is possible that removal of these cells could limit the number of spores and reduce recurrence resulting from endogenous spores that survive in the gastrointestinal tract [191]. Recently, Hurdle *et al.* [78] investigated this hypothesis showing that effective killing of stationary phase *C. difficile* was achieved by membrane-active reutericyclins, within several hours at concentrations close to their MIC, in contrast to the anti-difficile agents vancomycin and metronidazole. Further studies also showed that stationary cells could be killed by ionophores (e.g., nigericin), membrane disrupters (e.g., nisin), and the ATP synthase inhibitor *N*,*N*′-dicyclohexylcarbodiimide (DCCD), suggesting that the membrane and associated bioenergetics might be an Achilles heel in *C. difficile*, which mirrors observations with *M. tuberculosis* [77, 192]. Interestingly, oritavancin and CB183,315, an analog of daptomycin, are in clinical development for CDI. In the hamster model of CDI, oritavancin at 50 mg kg^{-1} was more efficacious than vancomycin (50 mg kg^{-1}) as some 80% of infected animals survived with oritavancin treatment compared to only 40% with vancomycin [193]. Using an *in vitro* human gut model, the research of Wilcox and colleagues [194] supports the superior *in vivo* efficacy of oritavancin. In the gut model, oritavancin reduced both vegetative and spore populations below detectable limits within 2 days of treatment, and did not yield toxin recrudescence after the 7 days of treatment. In contrast, during 7 days of treatment, vancomycin was not effective in eliminating spores and posttreatment was associated with cell proliferation and toxin production. Several analogs of daptomycin also exhibit substantially improved efficacy over vancomycin; at 0.5 mg kg^{-1} only 25% of hamsters survived

with vancomycin, while survival with a cyclodecane and cyclohexyl derivatives were 100 and 60%, respectively [195]. The oral bioavailabilities of oritavancin and daptomycin analogs are low, making them well suited for localized *C. difficile* therapy.

8.12 Is Inhibition of Fatty Acid/Phospholipid Biosynthesis Also an Approach?

Given the essentiality of the phospholipid membrane bilayer to bacterial survival and response to environmental stress, it is tempting to speculate that the inhibition of fatty acid and phospholipid biosynthesis would affect dormant cells. Currently, there is a lack of evidence to support this assumption. Nevertheless, the bacterial FAS II pathway is receiving much attention for the development of novel antibiotics. This is evident from the continued discovery of natural product compounds such as platensimycin that inhibit Fas II enzymes [54] and the progress of the synthetic product AFN-1252 (Affinium Pharmaceuticals Ltd) in phase II clinical trials for acute bacterial skin and skin structure infections of staphylococci. Recently, Brinster *et al.* [196] debated the clinical usefulness of Fas II inhibition, as their work showed that a supply of fatty acids in human serum rescued *Streptococcus agalactiae* from inhibition with triclosan and cerulenin. This finding is unlikely to invalidate the Fas II pathway for antibiotic discovery, as differences in fatty acid metabolism occur between organisms. For example, the drug isoniazid, which targets the enoyl-acyl carrier protein reductase (FabI), has long been a key antitubercular drug, while several studies show that Fas II inhibitors are efficacious in animal models [197]. In addition, a study by Baleman *et al.* [198], demonstrates that *S. aureus* is not rescued by exogenous fatty acids and human serum, indicating the diversity of fatty acid metabolism in bacteria. Whether Fas II enzymes will emerge as targets that affect dormant bacteria remains to be seen, but there is ample evidence that isoniazid does not kill nongrowing *M. tuberculosis* and this appears to be due to a decrease in cell turnover and cell envelope synthesis [199]. The downregulation of Fas II within the metabolically inactive layers of biofilms might also suggest that this pathway is inactive in dormant cells and may not yield the same detrimental effects as seen with growing cells [39].

The phospholipid biosynthesis enzymes PlsB and ubiquitous PlsX/PlsY represent alternative targets to Fas II enzymes for discovering novel antibiotics [200, 201]. Both enzyme systems catalyze the acylation of glycerol-3-phosphate, which is the first step in the synthesis of phospholipids. The PlsB enzyme in *E. coli* has been extensively studied and is responsible for selecting the fatty acids to be incorporated into membranes [200]. Interestingly, a strain carrying a dysfunctional PlsB showed a significant reduction in the formation of persisters in the stationary phase, suggesting that PlsB could be a target for preventing persister formation. Inhibitors of phospholipid biosynthesis have been reported but their effects on the survival of dormant cells are unknown [201].

8.13 Concluding Remarks

The advent of membrane-active molecules as a new paradigm to control dormant infections is arguably one of the major chemotherapeutic advances since penicillin ushered in the golden era of antibiotic discovery. Their discovery has certainly opened new avenues for not only killing dormant bacteria but also understanding the physiology of these cells in metabolically inactive states. This emerging area is not without limitations, notably issues of specificity for some molecules and pharmacokinetics and pharmacodynamics for others. There is also limited knowledge on medicinal chemistry strategies to optimize nonpeptide membrane-active molecules that are potent against bacteria, but show some cytotoxicity to mammalian cells. Addressing these issues could make a difference as to whether membrane-active compounds are developed for topical or systemic applications. There is also a substantial need for studies that identify what proteins are affected when nonlytic molecules are accumulated within the membrane bilayer and subsequent validation of whether any of these proteins are needed to maintain dormant cells or could be used in the design of pathogen-specific molecules. As gram-negative bacteria are a real concern, there is a need to extend the membrane-targeting paradigm to these organisms and dormant *M. tuberculosis*. We anticipate that different forms of persistent bacterial infections, not covered herein, would be susceptible to killing by agents that can disrupt the pathogen's membrane integrity. Understanding the structure–activity relationships of molecules and defining whether membrane-embedded proteins and peptidoglycan could confer selectivity for bacteria are areas that will certainly prove critical for the future development of novel membrane-active molecules to control dormant infections.

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9
Bacterial Membrane, a Key for Controlling Drug Influx and Efflux

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9.1
Introduction

In gram-negative bacteria, the outer membrane (OM) is the external barrier that protects the bacterial cell against environmental stresses including chemical, biophysical, and biological attacks [1–3]. At the same time, this structure is directly involved in the efficient uptake of nutrients required for bacterial growth and life. The OM exhibits a complex organization of proteins and lipid components, showing an asymmetric bilayer containing the lipopolysaccharide at the external side and phospholipids constituting the inner layer of the membrane [1, 3]. This specific architecture is responsible for the permeability behavior; and several channel proteins are embedded in OM, contributing to the diffusion of a large variety of hydrophilic molecules. Recent reviews have extensively reported the structural and biophysical aspects of this interesting class of membrane transporters and especially the general trimeric porins and the OM channels involved in drug expel [4–9]. An important point is the conservation of special trimeric porins inside the phylum of gram-negative bacteria with the presence of the eyelet region [10, 11]. This may reflect a well-designed system that effectively controls the diffusion of nutrients and limits the penetration of toxic compounds. Several aspects regarding the physicochemistry properties of porin function, for example, voltage gating and high stability of trimeric assembly, remain quite unclear when the key role of this class of transporters is of medicinal/pharmaceutical importance. The permeability properties of this barrier, therefore, have a major impact on the susceptibility of the bacterial pathogens to antibiotics, which are essentially targeted at intracellular processes [1]. Small hydrophilic drugs, such as β-lactams and fluoroquinolones, use the pore-forming porins to gain access to the cell interior, while macrolides and other larger hydrophobic drugs diffuse across the lipid bilayer. The existence of drug-resistant strains in a large number of bacterial species owing to modifications in the lipid or protein composition of the OM highlights the importance of the OM barrier in antibiotic susceptibility [1, 11]

Enterobacteriaceae, most notably *Escherichia coli*, *Enterobacter* spp., and *Klebsiella pneumoniae*, are among the most important causes of severe nosocomial and

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Table 9.1 Membrane permeability and intrabacterial antibiotic concentration.

Bacterial strategy	Mechanism	
	Influx (in)	**Efflux (out)**
	Passive diffusion	**Active transport**
Modification of membrane components (presence, production level, structure)	Porins (alteration of porin synthesis, mutation in the channel) LPS (modification of structure)	Efflux pumps (overproduction, expression of various efflux pumps)
Antibiotic classes altered by the bacterial change	β-Lactams, quinolones, and so on	β-Lactams, quinolones, cyclines, and so on

community-associated bacterial infections in humans. Consequently, resistance of these bacteria to antimicrobial drugs is a serious concern [12–14]. Of particular concern is the development of resistance to the carbapenem group, for example, imipenem, meropenem, ertapenem, and doripenem, because these drugs represent currently the last line of effective treatment available for infections with multiresistant *Enterobacteriaceae* in hospital wards [15–17]. This involves the emergence of specific carbapenemases [18–20]. It is important to note that the presence of plasmid-encoded carbapenem resistance corresponds to only a fraction of carbapenem resistance and cannot explain the resistance observed in the nonenzyme-producer clinical isolates [19]. In addition to this enzymatic barrier, many of the alterations in OM permeability described in clinical strains are associated with increased levels of antibiotic efflux [8]. Intrinsic antibiotic resistance is likely to reflect the synergistic action of the OM acting as a permeability barrier, and of the diverse and widely distributed efflux pumps (Table 9.1). The two mechanisms conjointly regulate the intracellular concentration of antibiotics (see Figure 9.1 and [21]) and consequently control the time/rate necessary to reach the threshold that triggers the antibacterial activity of one antibiotic.

$$C_{\text{act}} = I_{\text{in}} - \sum (E_{\text{ef}} + M_{\text{en}})$$

- C_{act} corresponds to the intracellular concentration of active antibiotic.
- I_{in} corresponds to the efficiency of antibiotic penetration. It depends on antibiotic structure (size, charges, etc.), penetration ways (channels, lipids, etc.), membrane physiology (growing cells, biofilm, or planctonic, etc.), bacterial species (e.g., *Enterobacteriaceae, Pseudomonaceae,* etc.) [2].
- E_{ef} corresponds to the rate of expel by efflux transporters. It depends on the activity of efflux pumps, pump type, structure of antibiotics, and affinity with pump sites [8, 22].

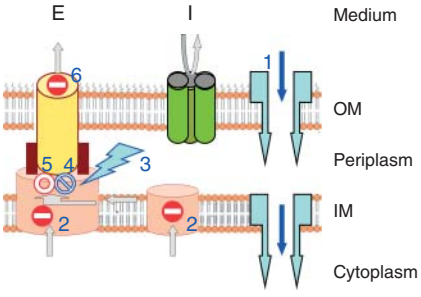

Figure 9.1 Acting on influx (I) or efflux (E), are two possible ways to improve the intracellular concentration of antibiotics. The gray arrows illustrate the impermeability (e.g., block of green porin channels in (I) or the efflux translocation by efflux pump (in (E)). In (1), the penetration of the drug can be improved by the addition of "membranotropic agent" (blue arrows) that permeabilizes the membrane barrier favoring the accumulation in periplasmic space or in cytoplasm. In (2), the use of specific plugs can induce steric hindrances in the pump cavities impairing the binding or transport of the drug. In (3), specific poisons (blue lightning) dissipate the energy source of the transport and stop the pump activity. In (4), some appropriate lures, mimicking the drug pharamacophoric elements, compete with selective sites, and are translocated in place of the active antibiotic. In (5), specific compounds can block the dynamic/mechanic of the pump according the dynamic model previously proposed (for a recent review see [8]). In (6), a specific plug can penetrate inside the outer membrane channel and block the final release outside.

- M_{en} corresponds to the level of enzymatic alteration of the antibiotic molecule. It depends on the enzyme expression, the enzyme affinity for the antibiotic, the presence of an enzyme inhibitor, and so on [2, 15].

This review essentially focuses on the key role of membrane transporters in the permeability changes and their role in the uptake of antibacterial agents. In order to discuss this topic, we have selected gram-negative bacteria. Whether changes in OM lipid or porin composition also mechanistically influence the efflux systems involved in expelling the drugs remains to be determined.

9.2
The Mechanical Barrier

9.2.1
The Outer Membrane Barrier and Porin Involvement

Substantial reports have described nonspecific porins in various gram-negative bacterial species such as *E. coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, and so on, with an abundancy corresponding to about 10^5 porin copies per bacterial cell, the most expressed OM proteins with OmpA and lipoproteins [1, 11]. These general porins form water-filled channels through which hydrophilic solutes gain

access into the bacterial cell [1]. In *E. coli*, the OmpC/OmpF balance is controlled by a complex regulation cascade involving the TCS EnvZ-OmpR, hns, micF, micC, and so on [23–26]. The OmpF family displaying a larger pore is present at low osmotic strength and low temperature (e.g., in water, effluent). The OmpC expression, favored by the conditions existing in the patient body (salt concentration, temperature), is a key for drug penetration and its selective expression seems to be an appropriate strategy for decreasing antibiotic susceptibility [11].

The study of porin expression in clinical isolates (type of porin expressed, level of expression, and regulation and association with a resistant phenotype) is complex because of the number of genes and external factors involved. In addition to local regulators that directly control the expression of porins, several global regulators (e.g., MarA, SoxS, and RamA) that are involved in the multidrug resistance (MDR) response, can strongly affect the synthesis of porins by regulating local effectors [2]. This sophisticated regulation reported in a lot of MDR clinical isolates allowed the bacteria to control influx and efflux of antibiotics [1, 2]

In *Enterobacter*, *Klebsiella*, and *Salmonella*, the Mar locus and RamA are two major MDR regulatory pathways. Mutations in the corresponding genes induce the overproduction of efflux pumps and inhibit porin expression both directly and indirectly [27–29]. In addition, various compounds such as salicylate, chloramphenicol, imipenem, and tetracycline are able to activate these and other unknown MDR regulatory pathways [30–35]. This phenomenon has been observed when bacteria are grown in the presence of subinhibitory concentrations of drugs and in hospital wards during antibiotic treatment of infected patients. These mechanisms are reviewed in detail in Davin-Regli *et al*. [2]. The 3D structure of OmpF and OmpC porins has been determined [36, 37]. A key feature is the internal loop (L3) that folds back into the channel to form the conserved constriction zone or ''eyelet.'' The electrophysiological studies of porins have been largely discussed in recent reviews [4, 11, 38]. Briefly, depending on the organization of the eyelet, porins exhibit various properties regarding the conductance, ion selectivity, and voltage closure and these parameters depend on the channel organization [11]. By studying the antibiotic diffusion, it is possible to dissect the electrostatic interaction between the passing antibiotic and the channel [39, 40], and dynamic simulations have also pinpointed on the energy level required during translocation through key points inside the channel, for example, the constriction area [40]. Several models have been proposed to illustrate the relationships between the energy level inside the channel and the antibiotic journey through the pore eyelet [39, 41]. The role of porins and specifically the general porins, in the antibiotic susceptibility has been discussed in a recent review [11]. The structure of the channel around the constriction area creates an eyelet with an intense electrostatic field (for a review, see [11]). The amino acid residues located inside this constriction have been selected during the evolution of bacteria and constitute a first screen for the orientation and diffusion of external solutes. The first demonstration of this role has been skillfully shown with the effect of mutation of charged residues highly preserved in the porin sequence, Asp and Glu (113 121). The replacement of these negatively charged residues by a

neutral Ala have generated a significant increase in β-lactam susceptibility and a serious decrease in MICs [39, 41, 42]. Recent works have molecularly dissected the role of porins in drug diffusion through the OM. Interestingly, the intensity of the minimal inhibitory concentration (MIC) modification conferred by specific residues depends on the structure and charge of the antibiotic molecules, and a recent study reports the molecular simulations and dynamics of β-lactams inside the wild-type and mutated channel during the travel of the molecule from outside to the periplasmic space [39, 41]. These data illustrate the adaptive pressure that had governed the selection and preservation of these specific residues that filter the diffusion of charged solutes. These amino acids represent the first defense against the penetration of harmful compounds and support the pioneer investigations reporting the difference in β-lactam susceptibility depending on porin [1]. Because β-lactams are originally produced by and directed against microorganisms, this porin functional organization may derive from an ancestral innate defense system controlling the uptake of β-lactam molecules present in the bacterial environment. In addition, the intensive and sometimes inappropriate human use of antibiotics has generated several bacterial adaptations including selection of porin group, modulation of expression, mutations, and so on [1, 43].

Several mutations located in key regions of the porin channel and involved in the antibiotic penetration have been described. A clinical isolate of *E. aerogenes* was found to have a Gly → Asp substitution on the L3 loop of its major porin [44], which might lead to a distortion of the loop and further narrowing of the pore lumen, as in G119D of OmpF [11]. This mutant is characterized by a threefold decrease in porin conductance and a drastic reduction in cephalosporin sensitivity. It was found later on that this porin is Omp36, which is highly similar to *E. coli* OmpC [11]. This clinical isolate and two others from *E. aerogenes*, in fact, present multiple mutations in the porin gene, and are also highly resistant to cefepime, cefpirome, and imipenem [45]. Similarly, a series of antibiotic-resistant *E. coli* isolates was isolated from a patient during serial treatment with various antibiotics. The sequence of OmpC changed at three positions during treatment, giving rise to a total of four OmpC variants [46, 47]. Dynamics simulations suggest that perturbation of the transverse electrostatic field reduces cefotaxime passage through the pore, consistent with laboratory and clinical data [47].

9.2.2 Membrane Modification

Besides alterations of porins involved in the drug penetration, different modifications of bacterial envelope regarding lipid components have been reported in clinical isolates during or after antibiotic treatment. Among them, modifications of the lipopolysaccharide are described in *Enterobacter*, *Pseudomonas*, and *Acinetobacter* following a treatment that includes polymyxins or other antibacterial agents, and these changes can be associated with a change in the chemical

structure of lipopolysaccharides (LPS) [48]. Furthermore, certain bacterial strains have lipid A modifications that occur in a manner consistent with a blockage of the self-promoted uptake of polymyxin across the OM [49–51]. Interestingly, in some cases, the alteration in LPS can promote a modification of the stability behavior of the OM, as recently demonstrated in the case of *Burkholderia cepacia* [52]. However, at this moment, only a few clinical bacterial strains have been isolated, characterized, and clearly documented. A recent study [53] demonstrated that resistant clinical isolates of *Acinetobacter baumannii*, which exhibited a marked resistance to colistin and polymyxin B, contain some modifications of the LPS, certainly exhibiting a modified lipid A moiety. The importance of LPS structure has been previously reported in recent reviews [1, 49, 54, 55]. In addition, alteration of LPS structure may impair the functional assembly of OM proteins, affecting the porin task in membrane translocation [3].

9.2.3 Efflux Barrier

Numerous papers describing the involvement of drug efflux pumps in clinical isolates have been published during the past decades (for recent reviews, see [8, 22, 56–60]).

Several isolates exhibit a decrease in antibiotic susceptibility to a large number of chemically unrelated molecules following antibiotic treatment of infectious diseases [61–67]. This decrease is associated with the expression of drug efflux pumps (usually resistance nodulation division (RND)-type in *Enterobacteriaceae*) that pump antibiotics out of the bacterial cell [5, 68, 69]. Several studies report the involvement of efflux pumps in the fluoroquinolone resistance of *E. coli*, *S. enterica*, and *Shigella* spp.-resistant isolates [70–76]. The presence of active efflux pumps contributes also in a reduced susceptibility to macrolides in resistant *Campylobacter* isolates [77, 78].

It is worthwhile to mention that during clinical therapies of infectious diseases, several chemically unrelated antibiotics, for example, imipenem and fluoroquinolones, are able to select for the overexpression of efflux pumps in isolates colonizing patients [2, 5, 8, 69]. Moreover, efflux pumps are able to also expel biocides including disinfectants, antiseptics, sterilants, and preservatives that are frequently used in medical practice [5, 69, 79–82]. Currently, various reviews describe the involvement of AcrAB in the decrease in susceptibility of enterobacterial clinical isolates and dissect the mechanism by which the efflux pump system recognizes and translocates several antibiotics outside the bacterium [2, 5, 68, 69, 80]. The large polyspecificity of the AcrAB pump contributes to the process that triggers the emergence and dissemination of efflux-producing bacteria during the treatment of gram-negative bacterial infections. The significant increase in the number of bacterial isolates that overproduced efflux pumps is a marked illustration of their key role during bacterial infection and it reaches a worrying level [8, 68, 80, 83].

AcrAB-TolC overproduction impairs the activity of several antimicrobial agents, and to determine whether increased *acrAB* expression correlated with MDR, drug-resistance data collected at hospitals were analyzed in the case of *E. coli* isolates resistant to fluoroquinolones [84, 85]. Using the level of MDR that correlates the number of antibiotic classes for which the tested isolates are not susceptible, Swick *et al.* [76] report that, in general, the more severe MDR phenotype is associated with the overexpression of the AcrAB efflux pump. acrAB overexpression may be an interesting biomarker for MDR, taking into account the mechanism by which the bacteria become MDR [76]. Using an *in vitro* infection model that simulates human drug treatment, Singh *et al.* [86] dissected the interplay between two mechanisms of quinolone resistance and provided a new mechanistic framework in the development of high-level resistance: early low-level levofloxacin resistance conferred by AcrAB overexpression anticipated and induced acquisition of target site mutation(s) generating high-level resistance. Because fluoroquinolones are prescribed currently, there is an effective selective pressure for bacteria to become resistant to them and a direct efflux has been demonstrated for many quinolones [5, 69]. The gastrointestinal tract is a natural reservoir for *Enterobacteriaceae* and *E. coli* and this bacterial population is often associated with opportunistic infections. Consequently, during antibiotic treatment the selection, emergence, and accumulation of quinolone-resistant strains likely occurs in this niche. Consequently, it is not a surprise if a serious increase in the prevalence of efflux-producing *E. aerogenes* strains isolated in a French hospital during the past decade has been reported [87]. Recently, Lautenbach *et al.* [88] reported that about half of *E. coli* isolates exhibited an efflux pump overproduction. This frequency is higher than that of previous studies [89, 90], indicating that either the efflux mechanism may be becoming more widespread over time or the role of efflux pumps in bacterial resistance development may have been underestimated. Moreover, it is interesting to note that despite the many recognized substrates of efflux pumps, the presence of organic solvent tolerance was associated with a greater likelihood of resistance to chloramphenicol but not to other antibiotics [87, 88]. The emergence of clinical isolates overproducing AcrAB-TolC pump has been reported in *E. aerogenes* and *K. pneumoniae* by several teams [2, 91–94]. The involvement of active efflux in clinical isolates of *K. pneumoniae* has been reported and the majority of tested isolates successfully expelled a large part of penetrating ciprofloxacin [95].

Interestingly, the clinical relevance of efflux pump activity observed in resistant isolates and its involvement in the reduced susceptibility toward some β-lactams fits well with the kinetic analyses performed using the periplasmic hydrolysis of β-lactams [96]. The affinity for a specific antibiotic, based on the presence or absence of pharmacophoric groups involved in the pump–drug recognition process, is a key parameter in the efficacy of efflux [8]. The kinetics and dynamics aspects of pump–substrate interactions are discussed in several excellent reviews [22, 96, 97].

9.3
Circumventing the Bacterial Membrane Barrier

9.3.1
Increasing the Influx: Antibiotic plus Permeabilizer, "Increase I_n"

The first way to increase the intracellular concentration of antibiotics is to improve the molecular design in order to accelerate the diffusion rate (Figure 9.1). Several pharmaceutical companies have used this strategy to develop new molecule profiles, ensuring a faster penetration, for example, zwitterionic cephalosporins or fluoroquinolones [98] proposed to potentiate the activity of antibiotics by protecting the molecules against enzymatic attacks [99]. Some compounds have been designed to parasite other alternate uptake routes such as endogenous bacterial iron-uptake systems [100, 101]. To date, the successful clinical application of the concept, combination of antibiotics plus enzyme inhibitor, has been in the development and clinical use of inhibitors of β-lactamase activity in resistant bacteria. Clavulanic acid, sulbactam, and tazobactam are routinely used but none of these inhibits all of the four major classes of β-lactamases produced by clinically important bacteria [102].

9.3.1.1 Permeabilizers such as Polymyxins

Another strategy is focused on the use of a helper compound that promotes a better/faster penetration of the bacterial membrane barrier [99, 103]. These helper compounds have been derived from antimicrobial peptides or the polymyxin group [104]. With the toxicity associated with the polymyxin group, the reuse of these molecules as modulators of membrane permeability, or helper compounds, at low concentration defines an original concept in order to rejuvenate the activity of usual antibiotics impaired by the membrane barrier [103]. In this case, the use of membranotropic molecules exhibiting a reduced toxicity, for example, polymyxin B nonapeptide or other derivatives [104] is interesting. Several papers described the benefit obtained with this kind of combination tested *in vitro* on resistant isolates (for a review, see [103]). In addition, this is a combination of previously described molecules, and the type and ratio of each compound may be adapted according to the targeted bacterial species by using the defined pharmacokinetic/pharmacodynamic (PK/PD) references.

Regarding the combined therapy used during clinical treatments, numerous publications report the use of the colistin combination to treat resistant *Acinetobacter* or *Pseudomonas* infections when a more limited number described similar combinations including colistin against *Enterobacteriaceae* [105–107]. This colistin use is associated with the increasing level of resistance observed with the two former species and the failure of alternative chemotherapy [53, 108].

Some other cationic peptide antimicrobial agents have been recently described as potent membrane permeabilizers exhibiting reduced toxicity compared to polymyxin B [109]. In the group of octapeptins, battacin was recently described as having a depolarization effect on the cytoplasmic membrane and the capability to

kill resistant gram-negative bacteria [110]. This type of new antibacterial peptide could be used in a combination of antibiotics in order to increase their activity against MDR bacteria in future assays.

9.3.1.2 Natural Compounds

Currently, with the continuous dissemination of resistant isolates, several natural compounds have been assayed in order to restore the activity of usual antibiotic classes against resistant strains [111, 112]. In this context, carvacrol, a monoterpenic phenol, has emerged for its activity against bacteria including drug-resistant and biofilm-forming microorganisms [113]. The antibacterial activity of carvacrol has been attributed to its considerable effects on the structural and functional properties of cytoplasmic membrane and consequently it is able to select mutants exhibiting some changes in membrane lipid [114].

9.3.1.3 Silver Nanoparticles

With the development of nanotechnologies and nanoparticles (NPs), a new possible use of silver as a nanoparticle is proposed, the combination of nanosilver with usual antibiotics to treat MDR bacteria [115, 116]. Combining antibiotics and antimicrobial nanoparticles (e.g., silver nanoparticles) could be a promising approach to improve antimicrobial activity and potentially overcome resistance to the current antibiotics [117]. The field of nanomaterial-based or nanomaterial-assisted antibiotics (nanoantibiotics) is open [117] and the use of nanoparticle–antibiotic combination strategies have been discussed with regard to very few data available on the clinical applications and toxicity of NPs as antibiotics themselves and carriers of antimicrobial drugs.

9.3.2 Blocking the Efflux: Antibiotic plus Efflux Blocker, "Decrease E_{ef}"

Another major way to improve the intracellular concentration of antibiotics is to block the activity of bacterial efflux pumps (Figure 9.1).

During the past decade, the blocking of efflux pump activity has been discussed as a possible way to restore the intracellular concentration of antibiotics and to develop a new therapy using antibiotics combined with efflux pump inhibitors (EPIs) [57]. Various patents have been deposited and several reviews published regarding this new group of antibacterial compounds [57, 118–120].

With the scarcity of original molecules available in the pipeline of pharmaceutical companies, this alternative could be an attractive way to rejuvenate the activity of old antibiotics that are expelled by efflux pumps [121]. On the basis of a rational chemical synthesis using the structure of efflux pumps or the screening of a large library of compounds, several molecules have been identified during the past decades [57]. Regarding the development of blockers altering the activity of bacterial efflux pumps, various strategies can be mentioned (Figure 9.1):

- The use of specific plugs can induce steric hindrances in the entry of pump cavities, impairing the recognition step and binding of the drug.

- Specific poisons can dissipate the energy source of the transport and stop the pump activity.
- Some appropriate lures, mimicking the drug pharamacophoric elements, compete with selective sites and are translocated in place of the active antibiotic.
- Specific compounds can block the dynamics/mechanics of the pump according to the proposed dynamic model.
- A specific plug can penetrate inside the OM channel and block the final release outside.

Other possibilities exist: improving the molecular design of antibiotics by changing the pharmacophoric groups recognized by efflux pumps and responsible for the binding step, or using an ''escort'' molecule that masks the pump-affinity sites exposed at the surface of the antibiotic molecules in an escort–antibiotic complex [122, 123]. The latter way can roughly mimic the role of Qnr proteins that protect DNA gyrase and topoisomerase from quinolone inhibition [124].

It is plain that efflux blockers (modulators) reverse MDR by acting on the involved efflux transport, but, overall, the mechanism of inhibition is not well understood. Consequently, several molecules have been reported as efflux pump blockers but their mode of action, their precise targets, and the physicochemical parameters remain quite unclear [8]. In order to define a clear profile/behavior for this new class of ''adjuvants,'' it is now urgent to define the key parameters, similar to enzymatic definition, for example, affinity, IC_{50}, and so on. In addition, for some of them, debates about their precise activity on the bacterial membranes are open depending on the selected controls, bacterial strains and species, and methodologies used during their characterizations [103, 125, 126].

9.3.2.1 **The Chemical Response**

Taking into account these aspects, the search and development of specific molecules directed against efflux pumps in one specific bacterial species, or specific for restoring the accumulation of a specific antibiotic class in different bacterial species, or specific to block the activity of a defined pump in specific species, or other targets are the aim of several laboratories.

Different evidences indicate that in case of tripartite pumps, AcrAB-TolC or MexAB-OprM are the representative systems acting in the MDR gram-negative clinical isolates [5, 8]. Consequently, many efforts are concentrated on the search for compounds that tend to modulate the activity of these RND proteins. It is important to note that the activity of efflux blockers (modulators, EPI) depends on their intracellular (cytoplasmic or periplasmic) concentration, and consequently the diffusion of blockers through the OM is a key step [127]. It is reported that, depending on the antibiotic class and the type of pump modulators used, various discrepancies on the amount of EPI needed for restoration can be observed regarding the restoring level of antibiotic activity. In any case, the availability of isogenic derivative strains, devoid of active efflux pump is necessary to define a blocker effect on the pump and discriminate a possible side effect [57, 127]. Several quinoline derivatives have been synthesized and tested on resistant clinical strains [128–130]. Interestingly, with a same selected strain (from *E. aerogenes,*

K. pneumoniae, or E. coli), the reversal activity of pump blockers is different regarding the antibiotic tested, as previously reported for phenylalanine arginyl β-naphthylamide (PAβN), the first modulator [131]. PAβN and quinazoline derivatives exhibited different effects on the ciprofloxacin, sparfloxacin, and erythromycin susceptibility assayed in an *E. aerogenes* strain overproducing AcrB, also reported for *K. pneumoniae* and *P. aeruginosa* strains with other antibiotics [132, 133].

1-(1-Naphthylmethyl)-piperazine (NMP), presents a different activity on a collection of clinical isolates of *E. coli*, in particular regarding the macrolide resistance reversal compared to PAβN [134, 135]. Moreover, NMP displays a moderate activity in reversing MDR in *C. freundii*, *E. aerogenes*, *S. marcescens*, and *K. pneumoniae* clinical isolates. Its reversal effect on resistance depends on bacterial species and drugs, and is different from those observed with PAβN [136]. Thus, the selectivity/efficacy of efflux pumps and the activity of the respective EPIs on the degree of altered resistance are strongly interconnected. It is also interesting to mention that NMP and selective serotonin reuptake inhibitors reverse the efflux of various dyes, indicating that the molecule acts selectively on the pump transport [137, 138].

Another efflux pump blocker has emerged from studies carried out on the pyridopyrimidine family [139]. Among this group, the effect of D13-9001 is specifically associated with strains expressing MexB pumps. D13-9001 presents a maximum synergistic effect with aztreonam, a substrate well recognized by MexB; and a new method successfully demonstrated the MexB-specific inhibitory effect of D13-9001 [125, 126]. However, D13-9001 was not so efficiently active on ciprofloxacin susceptibility, which is a substrate of MexB, MexY, and other pumps. A debate is rising regarding the effect of PAβN versus D13-9001 on the inhibition of efflux activity and the membrane permeabilizing effect conferred by PAβN [125, 126]. The main problem with these new antibacterial compounds is caused by the dose used in order to block the drug efflux and the possible intrinsic effect of the molecule on the membrane stability: it has been reported that owing to their chemical structure and membrane affinity some compounds can have a permeabilizing effect on the bacterial membrane [57, 125, 126]. Reversal agents such as PAβN, D13-9001, and so on, may be useful when used in combination with a substrate of multiple efflux pumps such as aztreonam or ciprofloxacin and an ''influx'' effect must be documented in order to evaluate if the chemosensitizer acts on influx or on efflux.

Because various pump structures exist, it may be difficult to rationally design a ''magic molecule'' that can block all major pumps active in all gram-negative bacteria. However, developing an inhibitor that can be used in combination with an antibiotic agent, belonging to the same family, is essential. For instance, the use of quinoline derivatives provides *in vitro* interesting benefits to the fluoroquinolone activity against MDR isolates overexpressing efflux pumps [133]. A series of clinical isolates of *E. aerogenes* has been used to screen and characterize the activity of quinoline and quinazoline derivatives during the past decade (for a recent review, see [103]). These compounds have been selected for their structural similarities with quinolones, which are the main studied class of efflux substrates [5]. The behavior of these molecules has been checked on a collection of MDR

clinical strains expressing various resistance mechanisms to different antibiotics including efflux pumps, altered membrane organization, target mutations, and enzymatic barriers. Some derivatives increase the susceptibility to the quinolone, phenicol, and cycline antibiotics, all of which are substrates of efflux pumps of *E. aerogenes* [103, 133]. These molecules significantly stimulated the intracellular accumulation of radiolabeled antibiotics such as norfloxacin or chloramphenicol in efflux-producer strains. The variation in the activity of tested antibiotics observed in the presence of the various efflux modulators depends on the respective location of ligands (antibiotics and molecules) inside the AcrB cavity that can induce a steric hindrance for antibiotic transport, a competition for the same binding site between the antibiotic and the derivative, and a binding of the compound that alters the affinity of the antibiotic to its site [8, 57].

Phenothiazines are a different class of chemosensitizers; indirect effectors of antibiotic potency derivatives such as chlorpromazine or thioridazine have been proved to sensitize resistant bacteria to the antibiotic to which it was initially resistant [56]. By using the measure of ethidium bromide accumulation and efflux, Amaral *et al*. has studied the effect of various phenothiazines including chlorpromazine and phenothiazine on various strains of *Mycobacterium avium*, *M. smegmatis*, *S. enterica*, and *E. coli* that express efflux activity. In the presence of phenothiazine, a decrease in efflux of ethidium bromide was reported [140–143]. The primary target of phenothiazines is the enzymes involved in the generation of metabolic energy and they induce the generation of mitochondrial permeability transition associated with transmembrane potential dissipation and calcium release [144]. In *Mycobacterium tuberculosis*, a recent study reports that terahertz (THZ) alters membrane and consequently damages the cell-envelope integrity [145]. A possible explanation for their reversal activity of efflux-associated resistance may be the dissipation of the membrane energy associated with the alteration of membrane organization necessary to ensure the antibiotic transport [57, 142]. Consequently, it is now important to clearly define the bacterial mechanism targeted at low concentration of this group of chemicals during a combination protocol involving usual antibiotics plus phenothiazine compounds [141].

Similar to peptidomimetics, the study on phenothiazines will be attractive for developing new relevant blockers of pump activity [143] as this molecule class opens the way to tackle the drug efflux at the energy level.

9.3.2.2 **Natural Products as Efflux Modulators**

There is an ecological rationale that plants generate *de novo* various antibacterial agents in response to microbial attack to protect themselves from pathogenic microbes [146, 147]. Several papers reported the antibacterial activity of natural extracts on resistant strains from clinical isolates or laboratory strains selected under antibiotic pressure (for a recent review, see [103]). About gram-negative bacteria, despite the limited results available, some very interesting ways are open regarding the effect of such natural products against efflux-producer strains [103, 148].

Several plant extracts have been investigated and several of them demonstrated a strong effect on the antibiotic susceptibility of enterobacterial clinical isolates [142, 149–151]. The mode of action of the compound and the precise mechanism that restores antibiotic susceptibility were not well characterized and structure–activity relationship (SAR) studies are missing to determine the involved pharmacophoric groups. Other natural compounds have been assayed for their capability to restore antibiotic activity by altering the efflux pump in resistant isolates [103, 148]. Recently, artesunate, an antimalarial compound, was shown to increase the antibacterial effect of β-lactams against *E. coli* strains and this effect is associated with the expression of the AcrAB [152].

Regarding the essential oils obtained from various plants and herbs, several authors reported a noticeable effect – diverse combinations between various oils and usual antibiotic molecules demonstrate an increase of antibiotic activity on MDR bacteria [153–156]. These essential oils, similar to chemical blockers, can also have an effect by decreasing the efflux activity or increasing the antibiotic penetration via alteration of bacterial membranes [154, 157]. Moreover, in one study, the essential oil was efficient at modulating the bacterial resistance of *E. coli*, *P. aeruginosa*, and *A. baumannii* strains; these two species exhibit a strong membrane barrier to antibiotic uptake. In this case, composition determination of the essential oil indicated that the activity described comes at least in part from geraniol, which was not described before as a modulator of antibiotic resistance [155]. With essential oils, the main problem is that a minor component may act as a catalyst to improve a membranolytic potential of other molecules or may act in combination with other minor products to simultaneously alter the membrane energy, the membrane transporter efficiency, and/or the membrane integrity.

9.4 Conclusion

Faced with the continuous increase in MDR in gram-negative bacteria and the scarcity of new molecules in the pipeline, there is an urgent need to develop new agents, allowing us to engage a rational counterattack tackling the bacterial mechanisms of antibiotic resistance. The situation is especially worrying in the case of gram-negative bacteria exhibiting a sophisticated structure of cell envelope. Consequently, improved understanding of key parameters that are involved in an efficient penetration of antibiotics through the bacterial envelope and in the efflux activity pumping the antibiotics out of key multiresistant pathogens is absolutely necessary.

About the influx, if several molecules have been described as targeting the bacterial membrane, only a few of them have been studied as ''adjuvant,'' except now with polymyxins. Empiric therapy comprising combination with polymyxins to treat gram-negative pathogens infections is currently an approach for patients colonized by MDR bacteria and some recent reviews discussed this specific aspect

[105, 158]. Several derivatives of this polymyxin class have been reported by the group of Vaara [104] and the syntheses of molecules with less toxicity open a way to bypass membrane barrier in gram-negative pathogens. With the reuse of this family of antibiotics as adjuvant, the possible selection of a polymyxin-resistant variant showing envelope modification is also a critical point of combination therapy [159]. The increase in intracellular concentration of old antibiotics may be also stimulated by the combination with other membrane permeabilizers such as antimicrobial peptides [160]. The redesign of exposed-pharmacophoric groups located at the surface of antibiotics taking into account the features that promote fast penetration through the membrane barrier can be an alternate possibility. In addition, a recent paper [161] reported the functionalization of nanoparticles with ampicillin and the resulting compound exhibits a noticeable activity against *P. aeruginosa*- and *E. aerogenes*-resistant strains.

Regarding the efflux mechanisms, the question remains about the development of molecules that mimic the structure of a specific antibiotic molecule (via the use of appropriate pharmacophoric groups) in order to block the efflux mechanism and induce an increase of the antibacterial activity for a specific antibiotic class. This may be a key question for the development and selection of future adjuvants or chemosensitizers able to restore a significant antibiotic concentration inside the bacterium.

In a recent review, Manchester *et al.* [162] discussed the molecular determinants in efflux pumps substrates by using a couple of *H. influenzae* strains, parental strain and isogenic derivative lacking AcrB. Four key points are discussed: the location of antibiotic target (periplasm vs cytoplasm), irreversible binding on bacterial target, self-promoted uptake, and ion trapping. These key parameters strongly contribute to the antibacterial behavior of the molecule and favor their intracellular concentration [162]. These respective properties are directly associated, again, with pharmacophoric groups and also with the targeted bacterium. Alternate ways can be also proposed by using blockers of OM channel (e.g., TolC, OprM), but at this moment, no molecules have been described. For other ways, poisoning the transport energy or altering the functional assembly of the efflux pump, no results supporting a possible clinical way have been reported [103].

The increased level of overall understanding of drug influx and efflux is mandatory to develop new strategies and novel drug discovery efforts against MDR gram-negative bacteria. Molecules changing the barrier properties of the OM lipid bilayer itself or modulating the activity of efflux pumps can have a direct impact not only on gram-negative bacteria susceptibility but also on the fitness of bacteria. Consequently, their use favors a bacterial adaptation and the emergence of a new resistant generation. Whatever the selected ways, increasing the penetration or blocking the efflux activity, we must anticipate being ready to face the emergence of new resistances against the adjuvant/escort molecule used in combination. Appropriate investigations, from functional pharmacochemical to bacteriological-genetic, must be carried out to determine the best efficient combination, the targeted bacterium and the possible and future associated risk (selection and dissemination of resistant mutants).

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10
Interference with Bacterial Cell-to-Cell Chemical Signaling in Development of New Anti-Infectives

Jacqueline W. Njoroge and Vanessa Sperandio

10.1
Introduction

In the past three decades, bacterial communication has been widely accepted as an important facet of microbes' existence. Bacteria have the ability to sense chemical signals that may be self-produced or that are produced by other organisms in their environment. These signals known as autoinducers (AIs) allow the bacteria to interact with each other in a manner that may be intra- and/or interspecies, or interkingdom. The accumulation of these AIs, which is normally indicative of the bacteria's population density, allows for the coordination of gene expression and regulation in order to benefit the microbial community [1–3]. This phenomenon, known as *quorum sensing* (*QS*), was first observed in *Vibrio fischeri*, a bioluminescent bacteria that resides in the photophore, the light-producing organ, of the bobtail squid with which it has a symbiotic relationship [2, 4–6]. *V. fischeri* is able to sense the concentrations of the acyl homoserine lactone(AHL) AIs as they accumulate in the photophore, and when they reach a critical density, the photobacterium triggers the transcription of luciferase and subsequently light production.

Although QS is important for the coexistence of bacteria with its host, it has also been coopted by many bacterial pathogens for the tight regulation of the expression of their metabolically expensive virulence traits, such as the production of toxins and proteases as well as the formation of biofilms [7–11]. Biofilms, which are communities established when bacteria synthesize and aggregate within hydrated polymeric matrices, can adhere to both inert and living surfaces, and provide protection for their microbial inhabitants [12, 13]. QS coordinates the formation of these biofilms, which are inherently resistant to many antibiotics that the bacteria would otherwise be susceptible to in their planktonic form, leading to many persistent and chronic infections [11]. The fact that QS is a general mechanism of virulence gene control makes targeting it for the design of bacterial anti-infectives an attractive prospect. With the growing number of multidrug-resistant bacteria, targeting QS and bacterial signaling in general provides a nonconventional anti-infective strategy, which is usually nonlethal to bacteria, consequently significantly decreasing the potential to develop drug resistance.

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QS signals can diffuse freely into the cell where they interact with regulatory proteins, or they can be sensed by two-component systems (TCSs). In this chapter, we have divided the mechanisms used to target bacterial signaling and QS by how the signals are sensed, into TCS and non-TCS mechanisms.

10.2
Two-Component Systems (TCSs) as Potential Anti-Infective Targets

TCSs, which are absent in mammals, are composed of a sensor histidine kinase (HK) and a response regulator (RR). In response to an environmental signal, an HK autophosphorylates on a conserved histidine residue and then transfers the phosphoryl group to the aspartate residue of the RR [14]. The phosphorylated RR binds to the regulatory region of genes, which may encode for virulence traits, and activates or inhibits their expression. Bacterial pathogens can have only a few TCSs as is the case with *Helicobacter pylori*, the major cause of peptic ulcer disease [15–17], or a multitude of TCSs as observed in hemorrhagic-colitis-causing enterohemorrhagic *Escherichia coli* (EHEC) that has over 30 HKs and RRs [18]. This variation in the number of TCSs is thought to be an evolutionary response to the ecological niche of the bacteria and the competition present.

TCSs respond to single or multiple signals, which, other than QS signals, may include environmental cues such as nutrient levels, osmotic pressure, antibiotics, pH, and redox state. There is also cross-talk between TCSs, allowing for greater sensitivity [19]. TCSs control both nonpathogenic and pathogenic genetic clusters that include those that encode for cell growth, metabolism, division, biofilm formation, motility, and toxin production. Most currently used antibiotics work by targeting proteins that carry out functions essential for the bacteria's survival. In contrast, anti-TCS (potential) drugs do not target the virulence factors directly, but instead work by specifically inhibiting regulatory control functions [20]. This alternative targeting provides a number of major advantages. First, because these anti-TCS drugs are directed at hitherto untargeted mechanisms, it would make it possible to make new anti-infectives that are effective against various drug-resistant bacteria such as vancomycin-resistant *Enterococcus* (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA). Second, drugs that target nonessential TCSs, in particular, have the added advantage of inhibiting virulence with decreased evolutionary pressure toward development of drug resistance [21, 22]. Third, conserved TCSs have been identified not only in prokaryotic pathogens but also in eukaryotic pathogens such as *Candida albicans*, and these can be targeted to provide alternative, less toxic therapies [23, 24]. Lastly, HKs and RRs possess a high degree of active site homology [25], which suggests that multiple TCSs within a single bacterium, or a TCS found in many bacterial species could be inhibited by a single inhibitor leading to the development of broad range anti-TCS drugs.

In this section, we have divided the discussion on the molecular mechanisms of targeting TCSs into those TCS targets that are essential and those that are

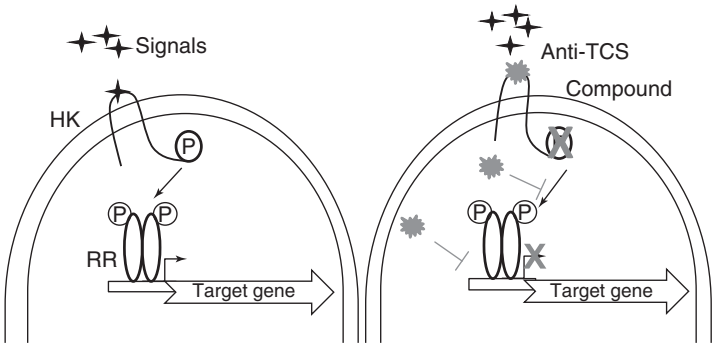

Figure 10.1 Inhibitors of two-component systems (TCSs). (a) Signals (black stars) sensed by the histidine kinase (HK) increase autophosphorylation of the HK and subsequently the phosphorylation of the response regulators (RRs) that bind to their target virulence genes to activate their transcription. (b) Anti-TCS compounds (gray) act by preventing (i) the binding and autophosphorylation of the HK, (ii) the phosphotransfer to the RR, and/or (iii) the binding of the RR to the promoter region of the target gene.

nonessential. Figure 10.1 summarizes the relationship between TCSs and their inhibitors.

10.3 WalK/WalR and MtrB/MtrA: Case Studies of Essential TCSs as Drug Targets

Several bacterial TCSs have been identified to be essential for the growth of pathogens (Table 10.1). Inhibiting their activity has been shown to interfere with functions that include cell-wall metabolism and the ability to replicate within macrophages [26, 27]. The WalK/WalR TCS (also known as YycG/YycF, VicK/VicR, or MicA/MicB), in particular, has been shown to be vital for cell-wall homeostasis in several gram-positive bacterial pathogens including *Staphylococcus aureus*, *Streptococcus pneumonia*, *Streptococcus mutans*, *Streptococcus pyogenes*, and *Staphylococcus epidermidis*. This TCS has also been shown to play a major role in *S. aureus* biofilm formation [28].

The WalK/WalR TCS was initially targeted by Qin *et al.* [31, 39], who utilized a structure-based virtual screen of a small molecule lead-compound library to identify WalK inhibitors. They identified two inhibitors with a thiazolidione core structure (compounds 2 and 5), which have bactericidal and biofilm-killing properties toward the opportunistic pathogen *S. epidermidis*. Since then, they have identified different derivatives of compound 2, which are more effective and less toxic, including some that inhibit the growth of planktonic *S. epidermidis* cells [31, 32]. Notably, these inhibitors not only displayed low cytotoxicity toward Vero cells (African green monkey kidney cells) and human erythrocytes but also displayed no obvious

Table 10.1 Essential TCSs and compounds that target them.

TCS (HK/RR)	Bacterium	Disease	Regulated gene	Function	Anti-TCS compound	References
WalK/WalR	*S.aureus*	Opportunistic infections	*IsaA*, *ssaA*, *lytM*	Cell-wall metabolism	Walkamycin B, Walrycin B	[29–32]
	S. pneumonia	Pneumonia	*PcsB*, *lytN*, *fabK*, *pspA*, *piaBCDA*	Cell-wall metabolism		
	S. mutans	Caries	*GtfBCD*, *fif* , *gbpB*	Biofilm formation		
	S. pyogenes	Necrotizing fasciitis	Unknown	Cell-wall metabolism		
	L. monocytogenes	Listeriosis	Unknown	Unknown		
	E. faecalis	Bacterial endocarditis, urinary tract infections	Unknown	Unknown		
	S. epidermidis	Opportunistic infections	Unknown	Cell-wall metabolism	Walkamycin B, Walrycin B, compound 2, compound 5	
YhcS/YhcR	*S. aureus*	Opportunistic infections	*NarG*, *nreABC*	Modulation of nitrate respiration	Unknown	[33, 34]
HP165/HP166	*H. pylori*	Chronic gastritis	HP1408, HP119	Unknown	Unknown	[35]
MtrB/MtrA	*M. tuberculosis*	Tuberculosis	*DnaA*	Replication in macrophages	ATB107	[36–38]

induction of hemolysis in these cells. Using natural products and a synthetic compound library, Okada *et al.* [29, 30] performed differential growth assays and homodimerization assays to screen for inhibitors of the HK WalK and the RR WalR, respectively. They identified two compounds, walkmycin B that inhibits WalK and walrycin that inhibits WalR, both of which showed antibacterial activity against MRSA.

Another essential TCS for which a potential targeting compound has been identified is the *Mycobacterium tuberculosis*' MtrB/MtrA TCS. The increasing emergence of multidrug-resistant tuberculosis (TB) and extensively drug-resistant TB strains in recent years highlights the importance of designing new anti-TB drugs [40]. The enzyme, indole-3-glycerol phosphate synthase (IGPS) is part of the tryptophan biosynthetic pathway that is absent in mammals, making it an attractive target for drug therapy [41]. The compound ATB107, which is a nitrogen heterocyclic ligand fused with polycyclic rings (Figure 10.2), had been shown to be a potent IGPS inhibitor, with the ability to inhibit the growth of not only drug-sensitive *M. tuberculosis* strains but also clinical drug-resistant strains [36]. Further investigation reported that the inhibitory effect on IGPS was due to the decreased expression of the RR MtrA [37]. Very little is known about the molecular mechanism

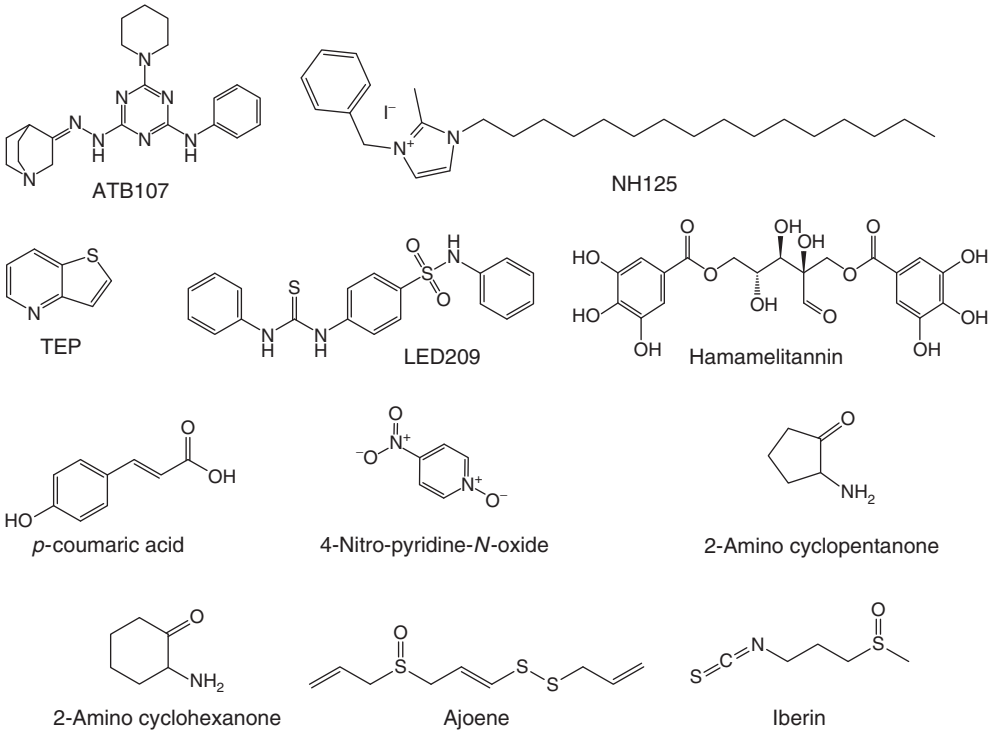

Figure 10.2 Chemical structures of anti-TCS compounds.

of ATB107's action on MtrA, or whether this inhibitor affects the HK MtrB's autophosphorylation activity; therefore, further evaluation of this compound is required.

10.4 Targeting Nonessential TCS

Upon entry into the host, bacteria need to adapt to environmental changes such as osmotic pressure, nutrient availability, and pH. They also need to evade the host immune system. In order to successfully colonize the host, pathogenic bacteria express virulence factors that include the production and secretion of toxins and proteases as well as mechanisms that evade the immune system. These virulence factors, however, are not required for the growth of the pathogen. Virulence factors are expressed in an energy-efficient and spatiotemporally efficient manner in response to a particular stimulus. These responses in bacteria have been shown to mostly rely on TCSs. Indeed, several TCSs in *Pseudomonas aeruginosa* are involved in virulence or antibiotic resistance [42]. The fact that a number of these TCSs are not essential suggests that targeting them for drug development would apply less evolutionary pressure toward drug resistance.

Synthetic nonessential TCS inhibitors were first reported in *P. aeruginosa* [43]. The AlgR2/AlgR1 TCS, which regulates the production of the exopolysaccharide alginate, an important *P. aeruginosa* virulence factor, was shown to have its function inhibited by several compounds including derivatives of isothiazolone and imidazolium. These compounds were shown to either inhibit the autophosphorylation ability of the HK AlgR2 and/or the ability of the RR AlgR1 to bind its DNA targets. These compounds were also shown to have an inhibitory effect on other kinases including CheA, NRII, and KinA. Since then, several compounds that affect a number of HKs in a single bacterium or in different bacteria have been identified (Table 10.2).

Later, we present the molecular mechanisms of several nonessential TCSs (Table 10.3) for which inhibitors have been identified.

Table 10.2 Compounds that target multiple HKs.

Compound	Histidine kinases inhibited	Bacteria inhibited	References
Imidazolium derivatives	CheA, NRII, KinA	*P. aeruginosa*	[43]
NH125 (imidazole derivative)	EnvZ, PhoQ, BvgS, EvgS	VRE, oxacillin-resistant *S. aureus*, Penicillin-resistant *S. pneumoniae*	[44]
TEP (thienopyridine)	WalK, HpkA, VanS, EnvZ	*S. pneumonia*, *E. faecium*, *E. coli*, *Thermotoga maritima*	[45]

Table 10.3 Nonessential TCSs.

TCS (HK/RR)	Bacterium	Disease	Regulated gene	Function	Anti-TCS compound (if known)	References
Human pathogens						
AlgR2/AlgR1	*P. aeruginosa*	Opportunistic infections	*AlgD*	Alginate production	Isothiazolone and imidazolium derivatives	[43]
QseC/QseB[a]	EHEC	Hemorrhagic colitis, HUS	*FlhDC, ler, stx*	Motility, attaching- effacing (AE) lesions, Shiga toxin	LED209	[21]
Salmonella spp.	Colitis	—	—			
Francisella spp.	—	—	—			
AgrC/AgrA	*S. aureus*	Opportunistic infections	RNA III	Invasive factor	Apolipoprotein B, noncognate AIPs, RIP analogs	[46–49]
FsrC/FsrA	*E. faecalis*	Opportunistic infections	*GelE, sprE*	Protease	Siamycin I	[50, 51]
GacS/GacA	*P. aeruginosa*	Opportunistic infections	*RsmY, rsmZ*	Lipase, elastase, biofilm formation	Unknown	[52]
PhoQ/PhoP	*S. enteric*	Diarrhea	*Ugd, pbgD*	Cationic antimicrobial peptide resistance	GHL inhibitors, for example, radicicol	[53]
Plant pathogens						
GacS/GacA	*E. carotovora*	Soft rot	*RsmB*	Extracellular pectinase, cellulose, protease	Unknown	[54]
PehS/PehR	*E. carotovora*	Soft rot	*PehA*	Endopolygalacturonase	Unknown	[55]
CorS/CorR	*P. syringae*	Chlorosis	*Cfa*	Coronatine synthesis	Unknown	[56]
HrpX/HrpY	*E. amylovora*	Fire blight	*HrpL*	Type III protein secretion	*p*-Coumaric acid	[57]

[a] Showing only a subset of pathogens that have the QseC/QseB TCS; this TCS has been shown to be important for virulence in many pathogens.

10.4.1 QseC/QseB

The QseC/QseB TCS is conserved in many bacterial pathogens including EHEC, *Salmonella typhimurium*, and *Francisella tularensis* [21]. In the enteric pathogen EHEC, which causes hemorrhagic colitis and hemolytic uremic syndrome (HUS), antibiotic use is controversial as it can lead to the development of HUS [58]. The fact that current treatment for EHEC infections is supportive only highlights the need to identify therapeutics that downregulate EHEC virulence expression without stressing/killing the bacterium, which has been shown to be responsible for expression of the Shiga toxin that leads to HUS. The EHEC HK QseC has been shown to sense AI-3, a gut microbiota-produced signal, as well as host-produced hormones epinephrine and norepinephrine [59, 60]. QseC in turn phosphorylates its cognate RR, QseB, and also two other RRs, QseF and KdpE [61, 62]. These three RRs subsequently regulate the repertoire of EHEC virulence genes.

Rasko *et al*. [21] performed a high-throughput screen to identify chemical compounds that inhibit QseC's ability to respond to AI-3, epinephrine, or norepinephrine. They identified the compound LED209 (Figure 10.2), which inhibits the binding of these signals to QseC, subsequently suppressing EHEC's pathogenicity *in vitro* and *in vivo* without affecting bacterial growth. LED209 was also shown to be effective against *S. typhimirium* and *F. tularensis*. The fact that QseC homologs are found in many bacterial pathogens that affect mammals or plants makes LED209 a promising broad-spectrum therapeutic.

10.4.2 AgrC/AgrA

S. aureus is a gram-positive, opportunistic pathogen. It is responsible for a wide range of diseases from minor skin conditions, such as abscesses and impetigo to more life-threatening conditions like meningitis, pneumonia, food poisoning, endocarditis, toxic shock syndrome, and septicemia [63]. It is also one of the leading causes of nosocomial infections, which manifest as chronic wound infections. A major problem associated with *S. aureus* infections is the rise of MRSA and multidrug-resistant strains [64]. Newly developed drugs such as linezolid and daptomycin, which are currently being used to treat MRSA, have been shown to be ineffective against certain MRSA strains [65]. This highlights the importance of identifying novel therapeutics.

S. aureus has two phenotypes, an adhesive colonizer phenotype and a severe, invasive, infective phenotype, the latter of which is responsible for the majority of the disease manifestations [66]. The phenotype switch is mediated by the QS AgrC/AgrA TCS. The self-produced *S. aureus* signal, Autoinducing peptide (AIP), is sensed by the HK AgrC, which in turn transfers its phosphoryl group to the RR AgrA [67]. Phosphorylated AgrA downregulates the expression of surface adhesins while upregulating the expression of invasive virulence factors such as secreted toxins, proteases, and lipases [66]. The host lipoprotein, apolipoprotein B, has been

shown to inhibit QS by sequestering AIP1, the major form of AIP [46]. This protein provides a natural innate barrier against infection by *S. aureus*. AIPs can also serve as their own antagonists. Four AIPs have been identified in *S. aureus* strains, and these signals vary slightly in their sequence. They have been shown to selectively bind to their cognate AgrC receptor, with a natural QS inhibition occurring when there is noncognate interaction [47, 68]. It is therefore reasonable to propose that synthesizing peptides that have close sequence similarity to AIPs may provide an alternative way to treat *S. aureus* infections.

A second QS system, RAP/TRAP (RNAIII-activating protein/target of RNAIII-activating protein) also regulates biofilm formation in *S. aureus* [69, 70]. The AI, RAP, has been suggested to be sensed by the HK TRAP, which then activates the Agr system. A heptapeptide, RNAIII-inhibiting peptide (RIP), has been shown to inhibit TRAP phosphorylation, and consequently Agr expression by competing with RAP [71, 72]. Through structure-based virtual screening, a number of inhibitors were identified including a RIP-nonpeptide analog, hamamelitannin [48, 73] (Figure 10.2). This compound was able to inhibit RNAIII expression as well as prevent device-associated infections caused by MRSA *in vivo*.

10.4.3
FsrC/FsrA

Enterococcus faecalis is a gram-positive commensal that inhabits the gastrointestinal tracts of humans and other mammals, but is also responsible for opportunistic infections that include endocarditis, bacteremia, meningitis, and urinary tract infections particularly in nosocomial settings [74]. *E. faecalis* is resistant to many commonly used antibiotics, and the increase in multidrug-resistant and vancomycin-resistant strains continues to pose a serious clinical problem [75]. The FsrC/FsrA TCS positively regulates the production and secretion of a QS signal, gelatinase biosynthesis activating pheromone (GBAP), which is a cyclic peptide carrying a lactone ring [76]. The FsrC/FsrA TCS also senses this QS signal, leading to the expression of two virulence genes, *gelE* and *sprE*, which encode for the metalloprotease gelatinase and a serine protease, respectively [77].

Nakayama *et al*. [50] screened actinomycetal extracts for compounds that inhibited gelatinase and GBAP production in order to identify FsrC/FsrA inhibitors. They identified Siamycin I, a peptide antibiotic, which suppressed the transcription of the *fsrBDC* and *gelEsprE* operons. Siamycin I was later shown to specifically and directly inhibit the HK FsrC [51]. This compound was also shown to inhibit a range of activities including other HKs and adenosine triphosphate (ATP)ases.

10.4.4
PhoQ/PhoP

Salmonella spp. are major food-borne pathogens in humans and other mammals. The TCS PhoQ/PhoP, which responds to extracellular Mg^{2+} levels and antimicrobial peptides, is a major regulator of virulence in *Salmonella* and is estimated to

control almost 3% of this pathogen's genome [78]. PhoQ/PhoP has been shown to regulate *Salmonella's* antimicrobial peptide resistance, epithelial cell invasion, and intraphagocyte survival [79, 80]. Deletions of components of this TCS have been reported to be effective as vaccines in some species of *Salmonella* [81–83].

Because HKs and the GHL (gyrase, Hsp90, and MutL) family of proteins share a unique ATP-binding Bergerat fold [84], Guarnieri *et al.* [53] investigated the interactions of GHL inhibitors with the PhoQ catalytic domain by NMR chemical shift perturbation. An Hsp90 inhibitor, radicicol, was found to interact specifically with the residues in the ATP-binding pocket of PhoQ, and was also shown to inhibit PhoQ autokinase activity, albeit with a relatively low affinity. This study showed that GHL inhibitors and their derivatives may be useful lead compounds for the development of broad-range HK inhibitors. It is important to note that such inhibitors would have to be highly selective for HKs compared to mammalian enzymes such as Hsp90 with similar Bergerat folds.

10.4.5
HrpX/HrpY

Erwinia amylovora is a gram-negative plant pathogen, which causes fire blight in rosaceous plants such as apples and pears. Affected plants appear shrunken and blackened, which interferes with product quality and consequently threatens global food production [85]. *E. amylovora's* HrpX/HrpY TCS senses low nutrient levels, low pH, and low temperature, and subsequently regulates the secretion of phytotoxins and plant cell-wall-degrading enzymes such as endopolygalacturonase and pectate lyase through the type three secretion system (TTSS) [86]. Research into compounds inhibiting the *Erwinia* spp. TTSS identified *p*-coumaric acid (Figure 10.2), which was shown to reduce the promoter activity of TTSS structural components (*hrpA*, *hrpC*, and *hrpJ*) as well as TTSS secreted proteins (DspE, HrpW, and HrpN) [87, 88]. This was indirect evidence of a compound targeting the HrpX/HrpY TCS; therefore, further evaluation of this inhibitor's direct interaction with the TCS is warranted.

10.5
Non-TCSs Targeting Biofilm Formation and Quorum Sensing in *Pseudomonas* spp.

Pseudomonas aeruginosa is a gram-negative, motile, ubiquitous rod-shaped bacterium. Its high versatility allows it to tolerate a wide range of temperatures (4–42 °C), low oxygen, and minimal nutrient conditions. This adaptability allows *P. aeruginosa* to adhere and survive on hospital surfaces including medical equipment, culminating in nosocomial outbreaks characterized by general inflammation and sepsis. *P. aeruginosa* is an opportunistic pathogen and the major cause of chronic lung infections in cystic fibrosis patients and microbial keratitis (MK) in users of extended-wear contact lenses. This opportunistic pathogen also causes infections in immune-compromised patients including burn victims and those with HIV or

neoplasia. *P. aeruginosa* encodes a wide range of virulence factors, which include a TTSS, proteases, adhesins, and the ability to form biofilms [42].

Most of the knowledge concerning QS and bacterial signaling comes from research on *P. aeruginosa*. QS mechanisms have been shown to be important for *P. aeruginosa's* survival in the harsh conditions within the host and on surfaces, as well as a key component of how this opportunistic pathogen circumvents the host immune system to cause disease. Figure 10.3 summarizes the relationship between QS and this medically relevant pathogen. QS in *P. aeruginosa* relies on the production, release, and sensing of diffusible AIs, which are divided on the basis of their chemistry into two groups: AHLs and the 4-quinolones (4Q). These signals are produced and sensed by three QS systems, LasR-LasI and RhlR-RhlI for the AHLs, and PqsR-PqsABCDE for the *Pseudomonas* quinolone signal (PQS) signal [89, 90]. AHLs include *N*-3-oxo dodecanoyl-$_L$-homoserine lactone (OdHL) and *N*-butanoyl-$_L$-homoserine lactone (BHL), and are produced by the synthases LasI and RhlI, respectively [91]. PqsABCD synthesizes PQS [92]. These signals then bind to their respective transcription regulators/receptors and induce the expression of virulence and biofilm formation; OdHL binds to LasR, BHL binds to RhlR, while PQS binds to PqsR. In addition to being regulators of virulence and biofilm formation, *P. aeruginosa's* QS signals can also modulate the host response. PQS and OdHL have been shown to induce apoptosis in neutrophils and macrophages during MK infections, modulate dendritic cell activity, as well as divert TH1 differentiation toward TH2 cell differentiation *ex vivo* [93–95].

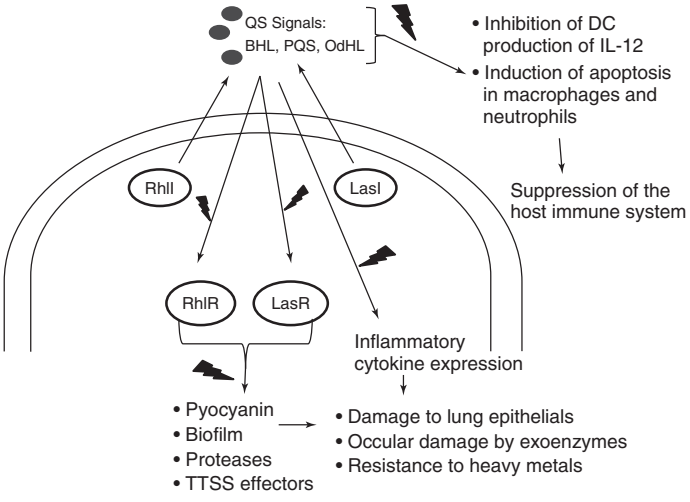

Figure 10.3 The relationship between QS and virulence in *P. aeruginosa*. Quorum sensing (QS) signal synthases RhlI and LasI produce signals (filled ovals), which are then released from the cell. These QS signals then diffuse back into the cells where they bind to the transcription regulators RhlR and LasR in order to activate virulence genes. The QS signals can also interfere with the host immune system independent of their transcription regulators. The black lightning symbols indicate potential therapeutic targets.

Targeting QS and biofilm formation in *P. aeruginosa* provides an alternative to conventional antibiotics. This alternative therapy, in theory, applies a gentler evolutionary pressure toward development of drug resistance because QS does not control processes essential for cellular survival and/or growth. Interestingly, subinhibitory concentrations of conventional antibiotics are thought to be able to induce or interfere with QS signaling and even promote biofilm formation [96].

Studies into *P. aeruginosa* QS and biofilms have identified a number of potential QS targets, as well as compounds that would interfere with their activities (Table 10.4). Gram-positive bacteria and eukaryotic cells have been shown to produce enzymes, such as lactonases and acylases, that are able to degrade AHLs [97–100]. In addition, compounds such as 4-nitro-pyridine-*N*-oxide (4-NPO)

Table 10.4 Compounds targeting QS and biofilms in *P. aeruginosa*.

Compound	Target	Mechanism of action	References
Lactonases, furanones and acylases, for example, PD12, V-06-018, C30, B7, 3oxo-C_{12}-acHone, 3oxo-C_{12}-acPol	LasR, biofilms	Bind to OdHL receptors and prevent their activation; degrade AHL signals; disrupt biofilm formation; increase susceptibility to antibiotics; destabilize LasR leading to LasR protein degradation	[97–100, 102, 106, 107, 109]
PPARδ agonists	PPARδ, OdHL	Bind to PPARδ and prevent the activation of NF-κB dependent proinflammatory genes	[110]
Ionic silver	Biofilms	High concentrations of ionic silver disperses biofilms	[111]
GA and DFO-Ga	Biofilms	Ga and DFO-Ga compete out Fe, an important cue for the initiation of biofilm formation	[112, 113]
NO-releasing silica nanoparticles	Biofilms	Silica nanoparticles provide a means for rapid diffusion of toxic NO for better biofilm dispersion	[114, 115]
4-Nitro-pyridine-*N*-oxide (from garlic)	AHLs, biofilms	Inhibits AHL biosynthesis; inhibits biofilm formation	[101]
Azithromycin	AHLs, biofilms	Inhibition of LasR-dependent gene expression; inhibits biofilm formation	[116–118]
Ajoene (4,5,9-trithiadodeca-1,6,11-triene 9-oxide)	Rhamnolipid, biofilms	Decreases rhamnolipid production; inhibits biofilm formation	[119]
Iberin (1-isothiocyanato-3-(methylsulfinyl)) propane	Rhamnolipid, LasIR, and RhlIR	Decreases rhamnolipid production; inhibition of LasIR-and RhlIR-dependent gene expression	[120]

(Figure 10.2) from garlic cloves [101] and halogenated furanones produced by marine alga *Delisea pulchra* [102] have been shown to also bind to LasR, thus inhibiting AHL binding and biofilm formation [102, 103]. Binding of furanones to LasR was also shown to result in faster degradation of the receptor, perhaps due to destabilization of its conformation [104]. However, further development of halogenated furanones as anti-QS therapeutics has been greatly limited by their toxicity, carcinogenic properties, and instability in aqueous solutions [105]. There have also been synthetic compounds identified that bind to LasR in an antagonistic manner, which include PD12, a tetrazole with a 12-carbon alkyl tail [106], 2-aminocyclohexanone, and 2-aminocyclopentanone (Figure 10.2) [107, 108].

Jahoor *et al.* [110] showed that OdHL can act as an agonist to perixome proliferator-activated receptor-β (PPAR-β) and PPARδ while acting as a PPARγ antagonist. PPARγ is a *trans*-acting repressor of the cytokine genes' transcription factor nuclear factor (NF)-κB [121], and by antagonizing PPARγ activity, OdHL is able to relieve the NF-κB trans-repression, consequently inducing apoptosis of macrophages and neutrophils [122, 123]. A PPARγ agonist, rosiglitazone, was shown to block the proinflammatory effect of OdHL in lung epithelial cells [110].

Recently, two compounds, ajoene and iberin, derived from the food products garlic and horseradish, respectively, were shown to inhibit QS [119, 120]. Iberin was reported to target both the LasR-LasI and RhlR-RhsI QS systems, and was also reported to regulate rhamnolipid production, which is a QS-regulated glycolipid that has been shown to cause necrosis in polymorphonuclear leukocytes [124]. The garlic compound, ajoene, was shown to have biofilm-killing properties and was able to clear *P. aeruginosa* infections in a pulmonary mouse model.

10.6 Conclusions

To date a significant number of lead compounds targeting bacterial signaling and QS have been identified, the majority of which target TCS. Some compounds, such as LED209 and walkmycin B, have been shown to target the same TCS in a number of pathogens, underscoring their potential as broad-range antimicrobials. However, there are still a number of pending issues. Although some of these lead compounds have been tested *in vivo*, most QS inhibitors still need to be evaluated in animal models of infection. Furthermore, although it is proposed that bacteria are less likely to become resistant to inhibitors that target QS without killing the bacteria, detailed studies comparing the rate of resistance evolution in QS inhibitors compared to conventional antibiotics need to be undertaken. Studies into treatment of drug-resistant pathogens with conventional antibiotics and QS inhibitors, such as ionic silver and DFO-Ga that disperse biofilms, could herald a new era of cotherapy. It may also be possible to develop highly specific ''designer'' drugs that specifically target a certain bacterial species without interfering with normal microbial flora of the host. Considering the tremendous potential of drugs that target bacterial signaling both as primary therapeutics or for cotreatment of

drug-resistant bacteria, it is of paramount importance to perform basic research to identify new signals and expand our knowledge of the ''bacterial language.''

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11
Recent Developments in Inhibitors of Bacterial Type IIA Topoisomerases

Pan F. Chan, Jianzhong Huang, Benjamin D. Bax, and Michael N. Gwynn

11.1
Introduction

The phase 2 and phase 3 pharmaceutical pipeline shows a concerning lack of new antibacterial agents in clinical development, at the same time as an alarming increase in antibiotic resistance [1, 2]. Medical, public, and governmental concern is exemplified by a call to action from the Infectious Diseases Society of America for a global commitment to develop 10 novel antibacterial drugs by 2020 [3]. Given the inevitable attrition experienced in drug development, and the wide range of pathogens to be addressed, new efforts are needed [4]. The disappointing productivity of molecular screening of new genomic targets has renewed interest in exploitation of clinically validated targets such as type II topoisomerases [5]. These are attractive targets for drug intervention [6] as they are clinically validated by the widely used fluoroquinolone antibacterials, inhibition is associated with bactericidal action, dual targeting of DNA gyrase and topoisomerase IV reduces emergence of resistance, and many different structural classes of inhibitors have been reported (several of which have progressed into clinical trials). Interestingly, many classes of type II topoisomerase inhibitors were discovered as antibacterials rather than from molecular target screens (e.g., quinolones, novel bacterial type II topoisomerase inhibitors (NBTIs), quinoline pyrimidine trione (QPT-1), gyramide, simocyclinone, clerocidin, novobiocin, and kibdelomycin), likely reflecting the strong potential for inhibition of this target class to result in appreciable antibacterial activity.

The double-helical nature of DNA can lead to topological problems when the two intertwined strands are separated for cellular processes such as replication or transcription [7]. Topoisomerases are responsible for resolving these problems by, for example, relaxing the positive supercoils that arise in DNA in front of the replication fork, and separating (decatenating) the two interlinked daughter chromosomes or plasmids that arise when circular bacterial DNA is replicated [8–10]. Type I topoisomerases regulate DNA topology by making a single-stranded break in DNA, while type II topoisomerases make a double-stranded break. Type IIA topoisomerases are structurally distinct from other classes of topoisomerases [8].

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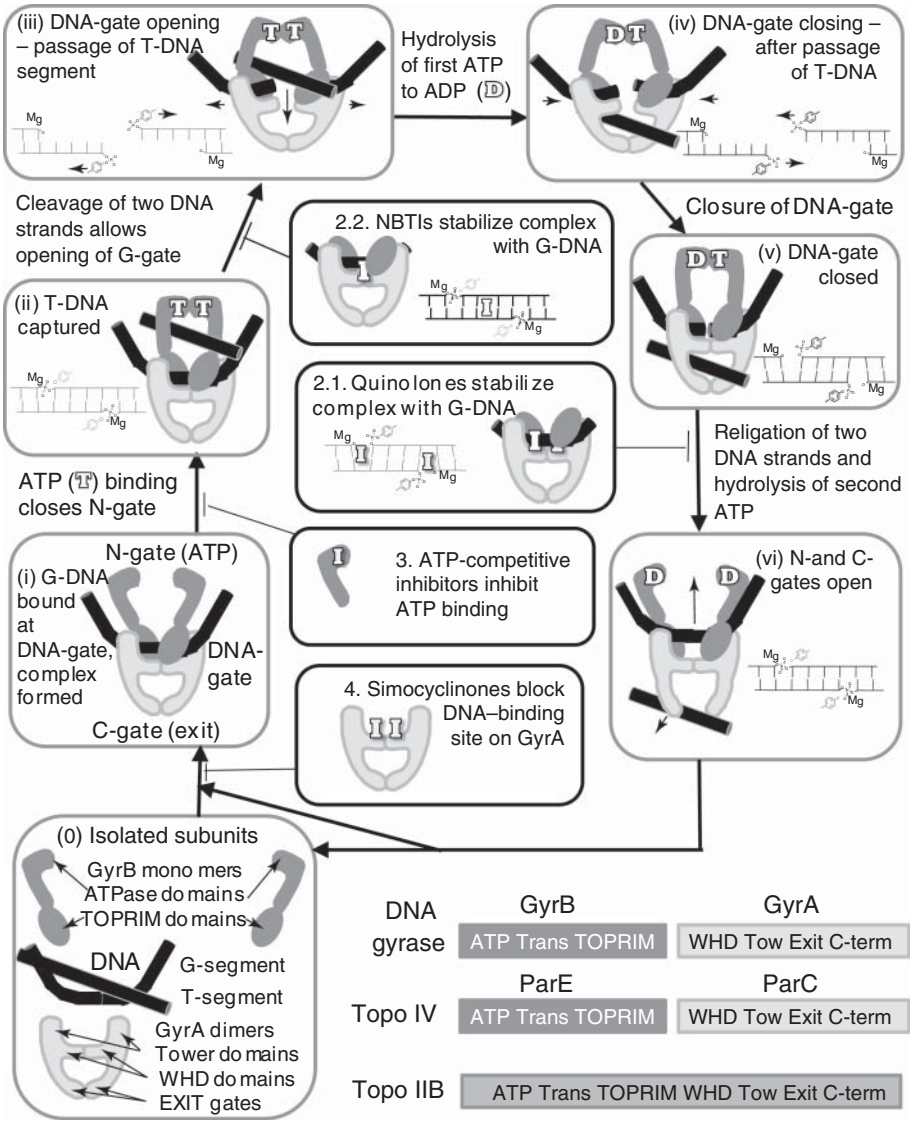
Most bacteria possess two type IIA topoisomerases, DNA gyrase ($GyrB_2GyrA_2$) and topo IV ($ParE_2ParC_2$), each of which functions as a tetramer (Figure 11.1). The two bacterial enzymes are quite closely related (sequence identity ~50%), enabling dual targeting [11]. In eukaryotic type II topoisomerases, regions equivalent to GyrB (or ParE) and GyrA (or ParC) are encoded at the N- and C-terminal ends of a single subunit, and the enzymes function as homodimers (Figure 11.1). The two type II topoisomerases in humans, topo IIα and IIβ, are closely related to each other (~80% identity), but less conserved with the bacterial enzymes (~20% identity between bacterial and human enzymes).

Bacterial type II topoisomerases regulate DNA topology by creating a four based-pair staggered break in one DNA duplex (the GATE or G-segment DNA), then passing another DNA duplex, the transport (or T-) segment through this break before religating the break in the G-segment DNA [8]. The G-segment DNA is bound and cleaved at the central DNA gate, where binding of the G-DNA brings together the catalytic tyrosine from GyrA and magnesium-binding residues from GyrB to form two catalytically competent cleavage sites, four base-pairs apart (Figure 11.1). Cleavage of the G-DNA is accomplished when the catalytic tyrosine residue from the GyrA subunit attacks the scissile phosphate, forming a phosphotyrosine bond and breaking the DNA backbone. The two DNA strands are cleaved successively to produce the four base-pair staggered break in the G-DNA segment. The passage of the T-DNA segment through the cleaved G-DNA modifies the topology of the DNA (Figure 11.1).

The N-terminal region of GyrB (ParE) contains an ATP-binding domain that dimerizes on binding ATP. The enzyme has a low basal ATPase rate, so that if the N-gate closes without the capture of T-segment DNA, the ATP will be hydrolyzed and the N-gate can reopen. However, capture of T-segment DNA brings the two ATP-binding domains closer together in a way that stimulates both N-gate dimerization (closure) and ATPase activity [12]. Structural studies [8] have shown that hydrolysis of the ATP can change the angle between the N-terminal ATP-binding domain and the transducer domain, which connects the ATP domain to the GyrB TOPRIM domain and the DNA-gate, helping to provide the energy to drive this molecular machine.

Figure 11.1 A simplified schematic view of the catalytic cycle of a bacterial type II topoisomerase (i–vi – around outside) and some inhibitor complexes (center – 11.2.1, 11.2.2, 11.3, 11.4). For clarity, the C-terminal (DNA-wrapping) domain has not been shown in the schematics of the enzyme cycle. In steps (ii–vi), the cleavage state of the G-segment DNA is illustrated by a line drawing of the central eight DNA base-pairs, with GyrA catalytic tyrosines and GyrB-bound Mg^{2+} ions also shown. Crystal structures of four different classes of inhibitor complexes are shown schematically in the center of the figure, with the approximate positions of *i*nhibitors represented by **I**. The numbers 11.2.1, 11.2.2, 11.3, 11.4 refer to sections of this chapter in which inhibitors are discussed. Note all crystal structures of inhibitor complexes, published to date, have been of truncated versions of the enzyme. The approximate positions of seven domains (*ATP*ase, *Trans*ducer, *TOPRIM*, *WHD* (winged helical domain), *Tow*er, *Exit* gate, and *C-term*inal) in DNA gyrase, topo IV, and a eukaryotic topo II are illustrated in the bottom right-hand corner of the figure.

The complex catalytic cycle offers multiple sites for drug intervention, reflected in the structural and mechanistic diversity of known inhibitors (Figure 11.2). Furthermore, inhibition of this DNA processing can have highly lethal consequences for the cell [7]. For convenience, in this chapter, we have grouped bacterial topoisomerase inhibitors into three categories: DNA-gate inhibitors – which are compounds that stabilize complexes with DNA (Figure 11.2a–j, Section 11.2), ATPase domain inhibitors (Figure 11.2k–r, Section 11.3), and inhibitors of DNA



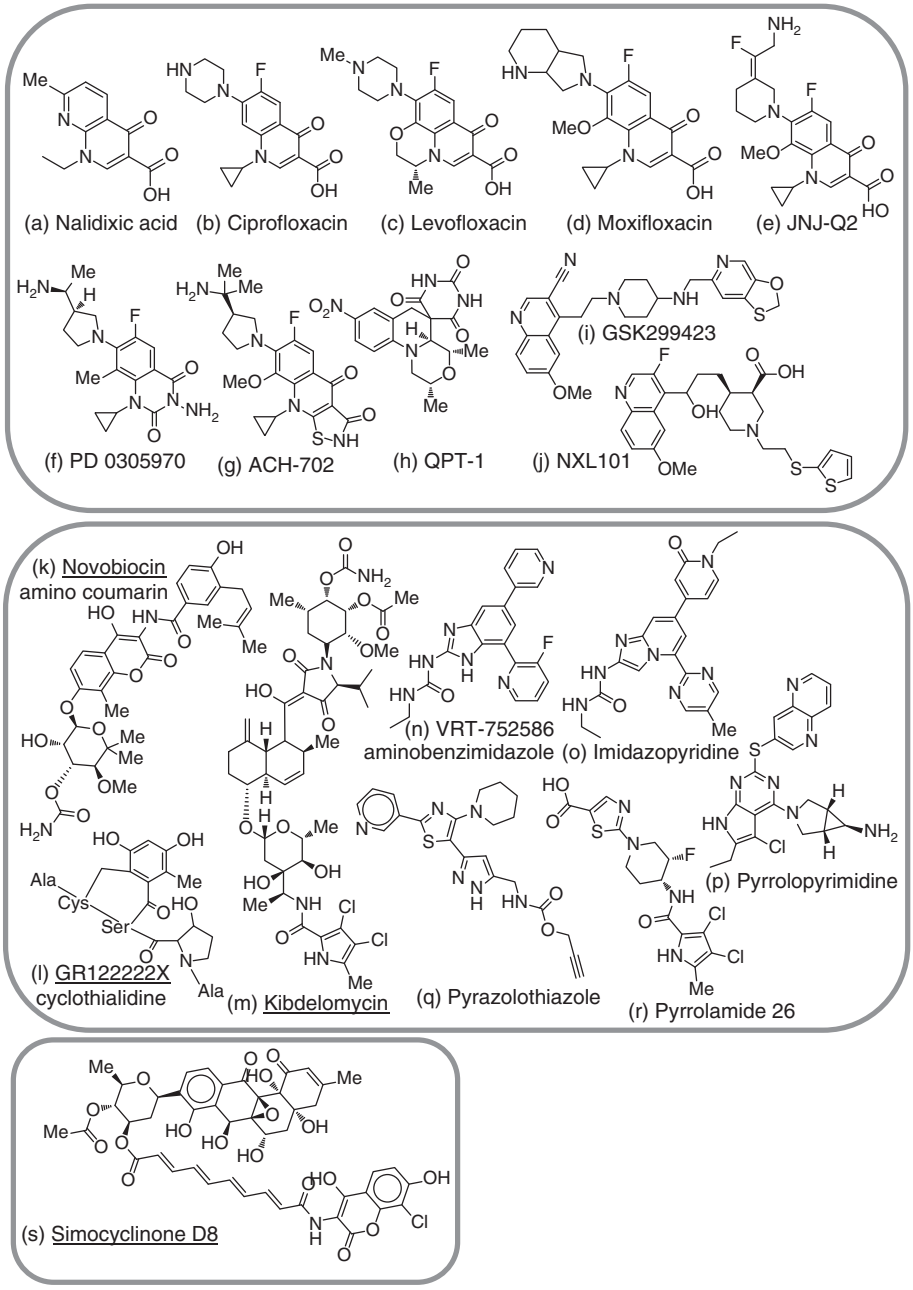


Figure 11.2 Chemical structures of bacterial topoisomerase inhibitors grouped by mechanism of action. (a–j) DNA-gate inhibitors, (k–r) ATPase-domain inhibitors, and (s) DNA-binder inhibitor. Natural products are underlined. Pyrimidoindoles (not shown) have structures similar to the pyrrolopyrimidine (p).

binding, such as simocyclinones (Figure 11.2s, Section 11.4). We have classified inhibitors into these three types based on available data and a comparison with structurally well-characterized inhibitors (see schematics in center of Figure 11.1). However, the structural basis of action of many inhibitors still remains to be fully characterized – so we cannot be certain that future experiments will not recategorize some inhibitors or even reveal new modes of inhibition of this complex and fascinating class of enzymes.

Topoisomerase inhibition is already a well-reviewed field [6, 13–16], and this chapter includes emphasis on recently reported inhibitors, and developments in the structural biology of the fluoroquinolones [17–20] and the novel NBTI class of inhibitors [20].

11.2 DNA-Gate Inhibitors

This section discusses inhibitors that stabilize complexes of G-DNA with the enzyme. These complexes can contain DNA that is double-strandedly cleaved, single-strandedly cleaved, or uncleaved (Figure 11.1 and subsequent text).

11.2.1 Quinolones and Related Compounds

11.2.1.1 Development of the Fluoroquinolone Class and Mechanism of Action

The quinolone family of antibiotics has been in clinical use since 1967, following the serendipitous discovery of nalidixic acid in 1962 as a by-product of chloroquine synthesis. Nalidixic acid (Figure 11.2a) was discovered by its antibacterial activity, and its targeting of DNA gyrase was later determined from resistant mutant analysis. Nalidixic acid was largely only used for urinary tract infection, owing to limited spectrum and potency, and its greatest significance was as progenitor for a new superfamily of antibiotics [21].

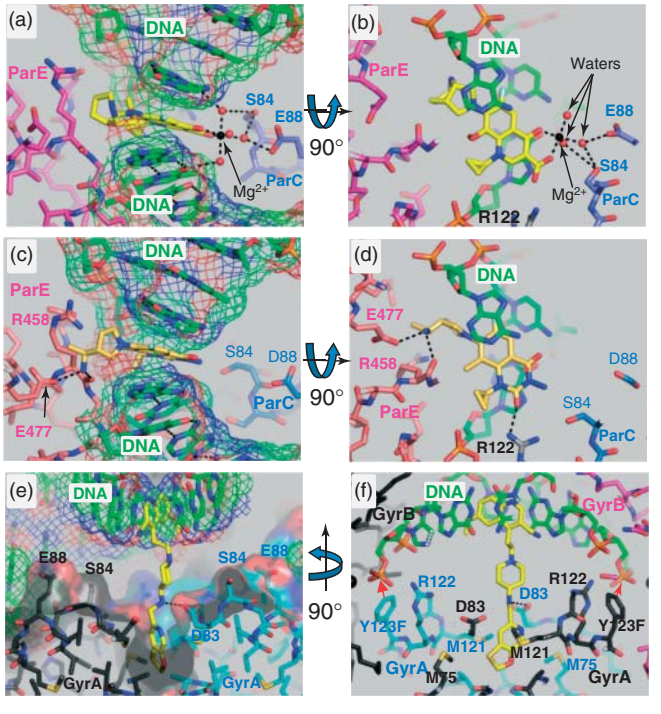
Several different quinolones are currently licensed for clinical use in the United States, representing different generations of improvements in spectrum and potency (e.g., first-generation nalidixic acid, second-generation ciprofloxacin and norfloxacin, third-generation levofloxacin and moxifloxacin, and fourth-generation gemifloxacin), establishing the class as among the most clinically and commercially important antibacterials [22–24]. Most used now are ciprofloxacin, levofloxacin, and moxifloxacin (Figure 11.2b–d) [23]. Quinolones are versatile agents, with oral, intravenous, and topical formulations, and are among few drug classes with a spectrum encompassing a broad range of gram-negative pathogens [2]. These successes have largely been achieved with empirical lead optimization approaches, as structure-guided approaches have not been available. The quinolones are still attracting considerable industry effort, and multiple compounds are in clinical development [25]. New indications are also being explored, with moxifloxacin in

clinical development for tuberculosis. Moxifloxacin may be one of the first new antibacterial agents approved for treatment of this disease in 30 years [26].

The attributes that have contributed to the durability and productivity of the quinolone class include good penetration into gram-negative pathogens, partly due to a particularly low molecular weight for antibacterials and good porin penetration [27]. While target-mediated resistance is a challenge to the class, its utility after over 40 years of use illustrates the benefit of dual targeting of topo IV and gyrase [28]. Resistance to fluoroquinolones usually occurs by mutation in target enzymes but also can occur by porin mutation and/or drug efflux [27, 28]. ''Dominant sensitivity'' appears likely to have prevented resistant target alleles to be spread on plasmids, a considerable advantage of the class [28]. However, after decades of use, plasmid-mediated quinolone resistance (PMQR) was discovered in 1998 [29], with the best-described mechanism mediated by the *qnr* group of genes, which codes for pentapeptide repeat proteins. In a way that is still not completely understood, these proteins protect the complex of DNA and type II topoisomerase (gyrase or topo IV) enzymes from the inhibitory effect of quinolones, and thereby reduce susceptibility of the organism to quinolones. An additional PMQR mechanism is based on drug inactivation. *aac(6′)-Ib-cr* encodes a variant aminoglycoside acetyltransferase, allowing it to inactivate ciprofloxacin through derivatizing its piperazinyl substituent. PMQR generally mediates relatively small increases in the MICs of quinolones, but can be sufficient to predispose to or exacerbate other forms of resistance.

Figure 11.3 Three crystal structures of DNA-gate inhibitors in complex with DNA and DNA gyrase or topo IV. (a,b) Two orthogonal views of the fluoroquinolone (FQ)-binding site in the 3.25 Å structure of moxifloxacin (yellow carbons) in complex with DNA (green carbons) and *A. baumannii* topo IV [17] (PDB code: 2xkk) (ParE magenta carbons, ParC blue carbons; R122 from the second ParC is in gray – two conformations). The residue numbers used throughout the figure are from *S. aureus* gyrase – numbers above SaGYRB and SaGYRA on sequence alignment (g). Note the interaction of the quinolone with S84 and E88 is via the Mg^{2+}-water bridge. (c,d) Two orthogonal views of the quinazolinedione (QD)-binding site in the 3.1 Å structure of PD 0305970 in complex with DNA and *S. pneumoniae* topo IV [19] (PDB code: 3ltn). (e,f) Two orthogonal views of the NBTI-binding site in the 2.1 Å structure of GSK299423 with DNA and *S. aureus* DNA gyrase. One covalently fused GyrB/GyrA subunit is shown with GyrB (magenta) and GyrA (blue); the other GyrB GyrA subunit is in dark gray [20] (PDB code: 2xcs – note in this structure the catalytic tyrosine has been mutated to phenylalanine, Y123F). Small red arrows indicate where the oxygen atom of the GyrA Y123 would attack the scissile phosphate to cleave the DNA. (g) Sequence alignment highlighting residues contacting the three different classes of DNA-gate inhibitors colored as: blue – contacts to the FQ moxifloxacin and the magnesium-water bridge, red – contacts to the NBTI GSK299423, and brown – contacts to the QD PD 0305970. Note that amino acid contacts to the three classes of inhibitors are distinct (except for residue R122 – colored in purple and underlined), this is despite the fact that two of the inhibitors, moxifloxacin and PD 0305970, bind at the same site in the cleaved DNA. There are no direct contacts to the catalytic tyrosine (Y123). The sequences shown are from: DNA gyrase and topo IV from two gram-positive (Sa = *S. aureus*, Sp = *S. pneumoniae*) and two gram-negative (Ab = *A. baumannii*, Ec = *E. coli*) bacteria. The DNA gyrase sequence from *Mycobacterium tuberculosis* (Mt) is also shown (Mt does not have a topo IV). Three eukaryotic sequences are also shown: human topo IIα and β (Hs = *Homo sapiens*) and yeast (Sc = *S. cerevisiae*) topo II.

The structural basis of quinolone action was unknown until 2009 [18], over 40 years after the first quinolone was used clinically, reflecting the challenge to crystallography from the complexity and flexibility of these molecular machines, with the added complication of co-crystallography with DNA. Only two groups [17–20] have thus far published on co-crystallography of quinolones or other inhibitors in ternary complexes with gyrase or topo IV and DNA. Bax *et al.* used protein constructs of gyrase and topo IV in which truncated subunits were translationally fused, comparable to native eukaryotic topo II. Structures using these constructs revealed the definitive fluoroquinolone orientation in its binding site, with a noncatalytic Mg^{2+} ion bridging between conserved residues on GyrA/ParC and the fluoroquinolone keto acid (Figure 11.3a,b). This is consistent with biochemical data that had implicated Mg^{2+} in quinolone binding [30]. The functionality and generality of this binding mode involving a water-Mg^{2+} bridge is



(g)

	456 460 474 478		67 75 84 88 120 123
SaGYRB	PLRGK NNNEI	**SaGYRA**	SARIVGDVMGK-YHPHGDSSIYEA AMRY
SpGYRB	PIRGK ANEEI	**SpGYRA**	SARITGDVMGK-YHPHGDSSIYEA AQRY
MtGYRB	PLRGK KNTEV	**MtGYRA**	SARSVAETMGN-YHPHGDASIYDS AMRY
AbGYRB	PLKGK SSQEV	**AbGYRA**	SARVVGDVIGK-YHPHGDSAVYET AMRY
EcGYRB	PLKGK SSQEV	**EcGYRA**	SARVVGDVIGK-YHPHGDSAVYDT AMRY
SaPARE	PLRGK KNEEI	**SaPARC**	SAKTVGDVIGQ-YHPHGDSSVYEA AMRY
SpPARE	PLRGK KNEEI	**SpPARC**	SAKSVGNIMGN-FHPHGDSSIYDA AMRY
AbPARE	PIRGK ASQEV	**AbPARC**	SARTVGDVLGK-YHPHGDSACYEA AMRY
EcPARE	PLKGK ASQEV	**EcPARC**	SSARTVGDVLGK-YHPHGDSACYEA AMRY
HsTOP2A	PLRGK ENAEI	**HsTOP2A**	VAQLAGSVAEMSSYHHGEMSLMMT SPRY
HsTOP2B	PLRGK ENAEI	**HsTOP2B**	VAQLAGSVAEMSAYHHGEQALMMT SPRY
ScTopII	PLRGK KNAEI	**ScTopII**	VAQLAPYVSECTAYHHGEQSLAQT AARY

supported by recent biochemical studies [31] which demonstrated the requirement for higher Mg^{2+} concentrations to support quinolone-induced DNA cleavage by quinolone-resistant ''S83'' (S84 in Figure 11.3a,b and Figure 11.4, which uses *Staphylococcus aureus* numbering) mutant enzyme. Furthermore, DNA cleavage induced by a quinazolinedione (a quinolone derivative lacking the keto acid group that binds Mg^{2+} eg. Figure 11.2f) was not Mg^{2+} concentration dependent [31].

The crystallographic studies answered fundamental questions regarding the molecular action and the mechanism of resistance of one of the most important classes of antibacterial drugs. Quinolones were shown to be inserted within the cleaved DNA at the two active sites, preventing DNA religation and stabilizing the enzyme-DNA phosphotyrosyl covalent complex (Figure 11.1, panel 11.2.1). The water-Mg^{2+} bridge (Figure 11.3a,b and Figure 11.4) explains why the keto acid is required in quinolone antibacterials and the mechanism of ubiquitous resistance mediated by S84 and E88 mutations. As the two amino acid residues that interact with the Mg^{2+} are not conserved in human topo II, it may at least partly explain selectivity for the bacterial enzymes compared to the human topo II homologs (Figure 11.3g, residues highlighted in blue). Quinolones were revealed to be interfacial inhibitors, with no direct protein interaction, but the inhibition mechanism is highly effective, and translates to strong ''kill-bug'' activity as a result of ''poison complex'' formation and induction of double-strand DNA cleavage.

However, the molecular structures do not fully explain the physiological basis of quinolone action, and the complex pathway(s) to cell death have yet to be elucidated. It has been long been known that the quinolones trap DNA gyrase and topo IV on bacterial DNA to form ternary complexes that inhibit activity of the

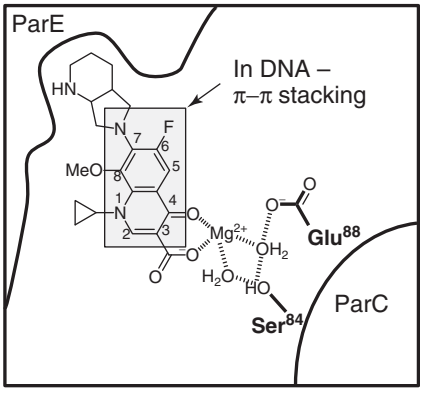


Figure 11.4 Interactions of moxifloxacin with DNA and topo IV. The interactions of moxifloxacin with DNA and topo IV in the 3.25 Å crystal structure (PDB code: 2xkk) [17] are illustrated schematically. Note that moxifloxacin has nonplanar constituents at positions 1, 7, and 8, making it more wedge shaped than a ''normal'' planar DNA intercalator. This seems to help the compound fit in the cleaved DNA and point oxygens on DNA bases toward waters that coordinate the quinolone-bound magnesium ion (Figure 11.3a). Nonplanar constituents at positions equivalent to 1, 7, and 8 are common in many related compounds (e.g., see Figure 11.2a–g).

enzymes and block DNA replication [32]. In bacteria, it was recently discovered that quinolone-induced formation of reactive oxygen species (ROS) further damages DNA to help promote cell death [33]. Additional pathway(s) to cell killing have been indicated by findings that moxifloxacin, for example, retains bactericidal action even when the ROS cascade is blocked or when oxygen is absent [34].

11.2.1.2 **Phase 2 Fluoroquinolones**

The fluoroquinolone family of antibacterials continues to show potential to address unmet medical needs and to attract considerable industry effort [25]. Examples in phase 2 clinical development include delafloxacin and JNJ-Q2 (Figure 11.2e) [35], both of which show promise against methicillin-resistant *Staphylococcus aureus* (MRSA). Delafloxacin, which recently successfully completed a phase 2b study [36], is a novel hospital-focused fluoroquinolone active against a variety of quinolone-resistant gram-positive and gram-negative bacteria, including quinolone-resistant MRSA [37]. JNJ-Q2 is a novel fluoroquinolone with broad-spectrum coverage, including potent *in vitro* activity ($MIC_{90} \leq 0.5\ \mu g\,ml^{-1}$) against fluoroquinolone-resistant MRSA isolates [38–40]. The novel C-7 group of JNJ-Q2 may contribute to additional interactions with GyrB/ParE (Figure 11.3 and Figure 11.4) giving higher affinity to the targets, which is supported by the findings that JNJ-Q2-passaged resistant strains have mutations in GyrB and ParE in addition to the commonly occurred ParC S80F and GyrA S84L mutations (equivalent residues); whereas only GyrA and ParC mutations were found for ciprofloxacin [38]. It would be interesting to solve the crystal structure of delafloxacin, JNJ-Q2, and other novel quinolones, in complex with gyrase or topo IV and DNA to give insight on the basis for improved target potency, especially the role of GyrB/ParC interaction.

11.2.1.3 **Quinazolinediones ("Diones")**

Quinazolinediones ("diones") represent a series of antibacterial agents, structurally related to fluoroquinolones, which challenge the dogma that 3-position acidic functionality is required for antibacterial activity in fluoroquinolones [41, 42]. A representative dione, PD 0305970 (Figure 11.2f), has exceptional antibacterial activity against gram-positive pathogens (including multidrug-resistant strains, especially MRSA with quinolone resistance). It inhibits both DNA gyrase and topo IV [43] with an $IC_{50} = 0.2\,\mu M$ against *Escherichia coli* gyrase, the same potency as for ciprofloxacin [41]. Similar to fluoroquinolones, PD 0305970 induces double-stranded DNA cleavage and is able to displace bound ^{14}C-labeled ciprofloxacin, suggesting a similar or overlapping binding region to fluoroquinolones. Spontaneous mutants resistant to diones were isolated from *Streptococcus pneumoniae* and *Neisseria gonorrhoeae*, and mutations were mostly found in the TOPRIM domain of GyrB and ParE instead of the more commonly occurring quinolone target mutations in GyrA and ParC [41–43]. Furthermore, purified dione-resistant gyrase (GyrB E474D or A) and topo IV (ParE E475A – both equivalent to E477 in Figure 11.3c,d) were resistant to dione inhibition in both enzyme inhibition and DNA cleavage assays (42), providing direct evidence that these mutations affect dione action at the target level.

One of the attractive features of the diones is their ability to overcome fluoroquinolone resistance. Isogenic quinolone-resistant mutants with gyrase and topo IV mutations constructed in *S. aureus*, *E. coli*, and *Mycobacterium smegmatis* showed no significant cross-resistance to diones, and in some cases were hypersensitive to diones [41, 44, 45]. Biochemically, quinolone-resistant enzymes isolated from *S. pneumoniae* (GyrA S81F and ParC S79F), *E. coli* (GyrA S83W, G81C, or A67S), or *Bacillus anthracis* (ParC S81F or S81Y) (all serine residues are equivalent to S84 in Figure 11.3) were shown to have little impact (generally less than twofold) on the enzyme inhibition and DNA cleavage with the diones [31, 43, 46], whereas dione-resistant gyrase and topo IV were similarly inhibited by quinolones such as ciprofloxacin as in wild-type enzymes [43]. The lack of cross-resistance between quinolones and diones can be explained by the recent crystal structure of a dione complexed with *S. pneumoniae* topo IV and DNA, indicating ParE interactions rather than ParC [19]. The S79 and D83 (labeled as S84 and D88 in Figure 11.3c,d,g) residues of ParC that are mutated in quinolone resistance are well removed from the dione N-3 amino group, whereas the conserved ParE R456 (R458 in Figure 11.3c,d), E474, E475 (E477 in Figure 11.3c,d), and D435 residues altered in dione resistance are clustered around the dione C-7 group (Figure 11.3c,d). This further highlights that the quinolone-binding pocket can be addressed with structurally diverse inhibitors to overcome fluoroquinolone resistance. However, no dione class lead has yet been advanced to clinical trials.

11.2.1.4 **Isothiazolones**

Heteroaryl isothiazolones (HITZs), are a class of antibacterials structurally related to quinolones, reported by Achillion, that display particularly good activity against *S. aureus*, including MRSA (Figure 11.2g). Mutational data shows that DNA gyrase is the primary target, with topo IV as a secondary target [47]. HITZs select mutations in the quinolone-resistance determining region, and DNA gyrase inhibition was associated with covalent complex formation, as also typical of quinolones. ACH-702 has shown promising *in vitro* activity against *M. tuberculosis*, including quinolone-susceptible and quinolone-resistant isolates [48]. ACH-702 is now reported to be in development for topical indications owing to metabolic instability of the inhibitor for systemic use [49]. The HITZs are a further illustration of the growing structural diversity associated with quinolone mechanism of action, and the potential to modify biological attributes including antibacterial spectrum and activity against fluoroquinolone-resistant strains.

11.2.2
"NBTIs," Novel Bacterial Type II Topoisomerase Inhibitors

NBTIs are a structurally and mechanistically novel class of antibacterials, first reported by GlaxoSmithKline in 1999 [50]. Biochemical and structural studies have shown that NBTIs bind and inhibit DNA gyrase and topo IV in a different way to fluoroquinolones, rationalizing a lack of target-mediated antibacterial cross-resistance [20, 51]. Their novelty, and targeting of well-validated fluoroquinolone targets

without cross-resistance, has attracted a lot of attention from antibacterial drug developers. Several thousand derivatives have been reported in patents and publications by multiple companies, including GSK [20, 52–54], Novexel [51], Actelion [55], Morphochem [56], AstraZeneca [57–59], Pfizer [60], J&J [61, 62], Toyama/Taisho [63], and Daiichi Sankyo [64]. The class encompasses apparently diverse compounds; however, crystallographic studies defining the target-binding mode have now provided unifying structural features [20], described in subsequent text.

NBTI inhibition of gyrase and topo IV is generally not associated with generation of double-strand DNA cleavage, as first revealed for NXL101 (Viquidacin) (Figure 11.2j) [51]. Studies on GSK299423 (Figure 11.2i) also showed absence of double-stranded DNA cleavage, although significant single-stranded DNA cleavage was demonstrable [20]. Both of these reports are consistent with NBTIs stabilizing a ''precleavage'' complex, in which the enzyme is trapped in complex with its DNA substrate at a stage of the catalytic cycle before double-strand DNA cleavage (Figure 11.1). This contrasts with quinolones, which stabilize a ''postcleavage'' complex resulting in double-stranded DNA breakage. However, both inhibitor classes have in common that they stabilize a ternary complex with enzyme and DNA. The NBTIs demonstrate that induction of double-stranded DNA cleavage is not a requirement for potent antibacterial activity for inhibitors stabilizing ternary complexes [20].

The structural basis of action of the NBTI class was reported by Bax *et al*. with a 2.1 Å crystal structure, an unprecedented resolution for this class of enzyme, showing GSK299423 in complex with an *S. aureus* gyrase construct and a 20 mer duplex DNA (Figure 11.3e,f) [20]. The NBTI is an interfacial inhibitor in not just one, but two, ways. It bridges between the DNA and a noncatalytic pocket in GyrA, and this ''GyrA'' dimer pocket is itself formed at the interface between the two GyrA subunits (Figure 11.3e,f). Interestingly, the pocket is absent in the apo-structure; and opens up at the GyrA dimer interface only when DNA binds, at which point this transient conformation is trapped by the inhibitor occupying the pocket, and the complex with DNA is stabilized. NBTIs, therefore, bind in between the two DNA cleavage-religation active sites (Figure 11.3f). Unlike quinolones, which bind in the cleaved DNA at the cleavage sites (Figure 11.1, panel 11.2.1) physically preventing DNA religation, NBTIs do not bind at the cleavage-religation sites (Figure 11.1, panel 11.2.2) and exactly how the NBTI-stabilized complex effects the DNA cleavage-religation equilibrium at the two active sites is still not fully understood. In the 2.1 Å crystal structure with GSK299423, the DNA is uncleaved and this represents the first precleavage structure with DNA for this class of enzyme. (Figure 11.3f – the small red arrows indicate where the oxygen atom on the tyrosine would attack the scissile phosphate to cleave the DNA). The GyrA/ParC binding pocket is very well conserved in bacteria [20] but not in humans (Figure 11.3g – NBTI pocket residues highlighted in red), likely reflecting that in bacterial apoprotein the pocket is closed, thus forming a buried protein interface. NBTIs with good activity against *M. tuberculosis* [65] tend to have smaller right-hand sides (RHSs) (see subsequent text), possibly because an alanine rather than a conserved glycine makes the pocket smaller in MtbGyrA (Figure 11.3g – position

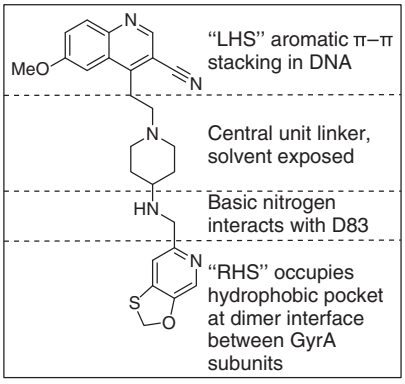


Figure 11.5 Interactions of NBTI GSK299423 with DNA and DNA gyrase. A schematic view of the structural features of NBTIs that are important for interactions with the binding pockets in the DNA and protein.

72). The elucidation of the respective binding modes of fluoroquinolones and NBTIs, as being adjacent but distinct, rationalized the lack of impact on NBTIs of target mutations that mediate fluoroquinolone resistance (Figure 11.3e shows positions of distal S84 and E88 residues implicated in quinolone resistance).

While the great structural diversity of NBTIs is known, the crystal structure with GSK299423 bound to DNA gyrase has identified functional features (Figure 11.5) that they all have in common: (i) an aromatic moiety that inserts by aromatic, π–π stacking in DNA (often called the "*left-hand side*" *LHS*); (ii) a highly variable central linker region that is exposed to solvent in between DNA and GyrA (ParC); (iii) a basic nitrogen that interacts with D83; and (iv) a moiety that occupies a relatively small hydrophobic pocket at the GyrA/ParC) dimer interface, often referred to as the "*right-hand side*." The NBTIs lack a single canonical structural feature such as a keto acid or a β-lactam ring, which characterizes certain other classes of antibacterials, but these topological and functional features characterize a coherent chemical class of antibacterial.

Does the NBTI class have the potential to match the medical significance of the fluoroquinolones? Time will tell. So far a few investigational compounds in the NBTI class have entered phase 1 clinical studies. NXL101 [51], one such investigational compound, was terminated because of QT prolongation in human subjects as a result of hERG inhibition [15]. Inhibition of hERG is a challenge for many classes of drugs, although many strategies are available to medicinal chemists to address this challenge [57, 59, 66, 67].

11.2.3
QPT (Quinoline Pyrimidine Trione)

QPT-1 is the first exemplar in a totally novel class of DNA gyrase and topo IV inhibitors (Figure 11.2h) discovered by whole-cell screening, with *in vitro* and *in vivo* activity against gram-positive bacteria and activity against efflux-deficient

mutants of hospital gram-negative pathogens [68–71]. Both *E. coli* gyrase and topo IV enzymes are inhibited by QPT compounds, but generally they have more potent activity against gyrase than topo IV. Similar to quinolones, QPT-1 induced double-stranded DNA breaks with good selectivity with respect to human topo IIα. The TOPRIM domain of GyrB was found to be the primary target, as spontaneous-resistant mutants were isolated from *S. aureus* and mutations mapped to GyrB (D437N/V or A439S). Commonly occurring quinolone mutations in the clinic (GyrA S84L and ParC S80F) have no effect on QPT MICs, suggesting interaction with different amino acid residues to quinolones. This new series illustrates that the quinolone mechanism of action can be accomplished by structurally diverse pharmacophores, and underlines the continued exploitability of the quinolone mechanism of action. Patent applications indicate that several hundred analogs have been synthesized by both Pfizer [72] and AstraZeneca [73].

11.2.4 Other DNA-Gate Inhibitors

11.2.4.1 Albicidin

Albicidin, a polyketide peptide antibacterial and phytotoxin of unknown structure, is a potent inhibitor of DNA gyrase with bactericidal activity against a range of gram-positive and gram-negative bacteria [74]. Mechanistically, albicidin strongly stabilized DNA gyrase cleaved complexes with a CC_{50} 30 nM that, unlike quinolone, required the presence of ATP. In addition, a GyrA S83L mutation in *E. coli* that conferred high-level resistance to quinolone gave only a fivefold increase in resistance to albicidin. The results suggest that albicidin is a DNA-gate inhibitor with a novel, but as yet uncharacterized, binding mode.

11.2.4.2 Clerocidin

Clerocidin, a microbial diterpenoid, is a potent inhibitor of DNA gyrase and mammalian topo II [75] with antibiotic activity primarily against gram-positive bacteria. Cleroocidin strongly promoted single- and double-stranded DNA cleavage with DNA gyrase [75] and topo IV [76]. A clerocidin-resistant mutant isolated in *S. pneumoniae* contained a novel mutation in GyrA G79A, which showed a 60-fold increase in clerocidin resistance but remained susceptible to ciprofloxacin [76]. These studies provide credence that gyrase is the primary target of clerocidin and that it modifies the DNA gate with a mechanism distinct from quinolone. Interestingly, clerocidin was also reported to have an unusual mechanism of action in that it differently modifies the two halves of the DNA gyrase gate [75].

11.2.4.3 Nybomycin

Nybomycin is a natural product described recently shown to be active against quinolone-resistant MRSA by a unique but poorly understood anti-gyrase mode of action [77]. Intriguingly, nybomycin enhanced DNA cleavage activity of *S. aureus* DNA gyrase mutated with GyrA S84L but not with the wild-type enzyme.

Furthermore, nybomycin-resistant mutants isolated in an *S. aureus* GyrA S84L quinolone-resistant strain were found to have *gyrA* genes back mutated to wild-type *gyrA*, leading to a loss of quinolone resistance. Understanding the structural basis of binding of this novel-acting antibiotic may open up new opportunities for overcoming quinolone resistance by combination treatment with nybomycin.

11.2.4.4 Macromolecular Inhibitors That Stabilize Complexes with DNA

Macromolecular inhibitors include microcin B17 [78], CcdB [79], and ParE [80] toxins, which are DNA poisons stabilizing covalent complex formation. This area has recently been thoroughly reviewed [13], including strides made in the structural basis of action. While beyond the scope of this review, these proteins/peptides further illustrate the structural diversity of bacterial type II topoisomerase inhibitors.

11.3 ATPase-Domain Inhibitors

Targeting the ATPase activity of bacterial type II topoisomerases represents a mechanism distinct from that of quinolones and an opportunity to overcome quinolone drug resistance. The discovery of natural antibiotics, including aminocoumarins (1950s) and cyclothialidines (1990s) as competitive ATP-binding inhibitors, together with the availability of high-quality crystal structures of GyrB and ParE, and the potential for finding dual targeting inhibitors of GyrB/ParE with a low propensity for resistance development has encouraged substantial research around the ATPase domain. Although many diverse structural classes of competitive ATP analogs that target the catalytic ATPase domain of GyrB/ParE have been discovered (Figure 11.2), novobiocin remains the only one that has progressed to the clinic. In the United States, novobiocin was marketed from 1957 as an antibiotic in six combinations with tetracycline and one combination with sulfamethizole, but certificates of safety and efficacy for these products were withdrawn by the FDA in 1969 [81]. A detailed discussion of all bacterial topoisomerase ATPase inhibitors is beyond the scope of this chapter, for some pre-2007 inhibitors we refer the reader to a review by Oblak *et al.* [14], and also to other recent review articles [13, 16]. Here, we have divided the discussion of ATPase inhibitors into two main parts, in Section 11.3.1, developments around three important classes of natural products are discussed, while in Section 11.3.2, recent novel, small molecule inhibitors developed in the past 5 years as a result of GyrB screening and structure-based docking efforts are reviewed.

11.3.1 Natural Products That Inhibit the ATPase Domain

11.3.1.1 Aminocoumarins

Aminocoumarins, produced by the *Streptomyces* species, are competitive inhibitors of the ATPase site of GyrB/ParE subunits, and encompass structurally related

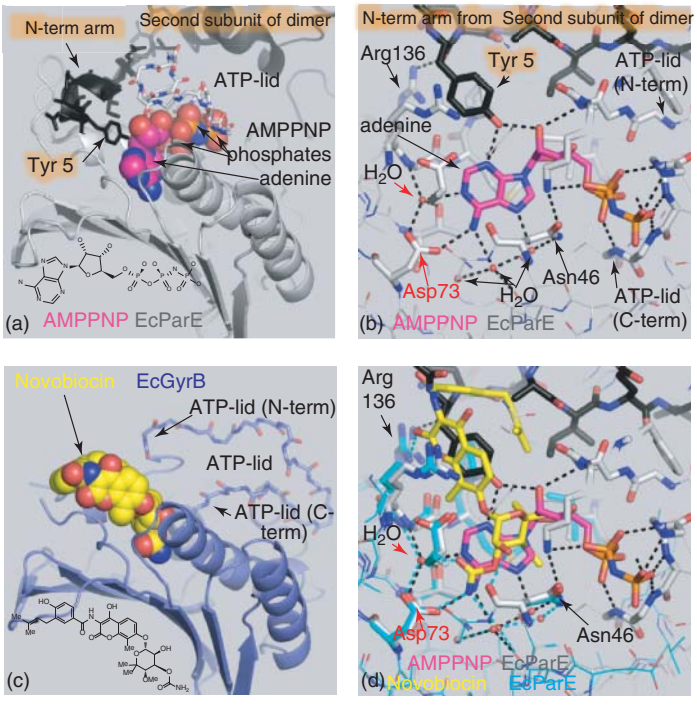


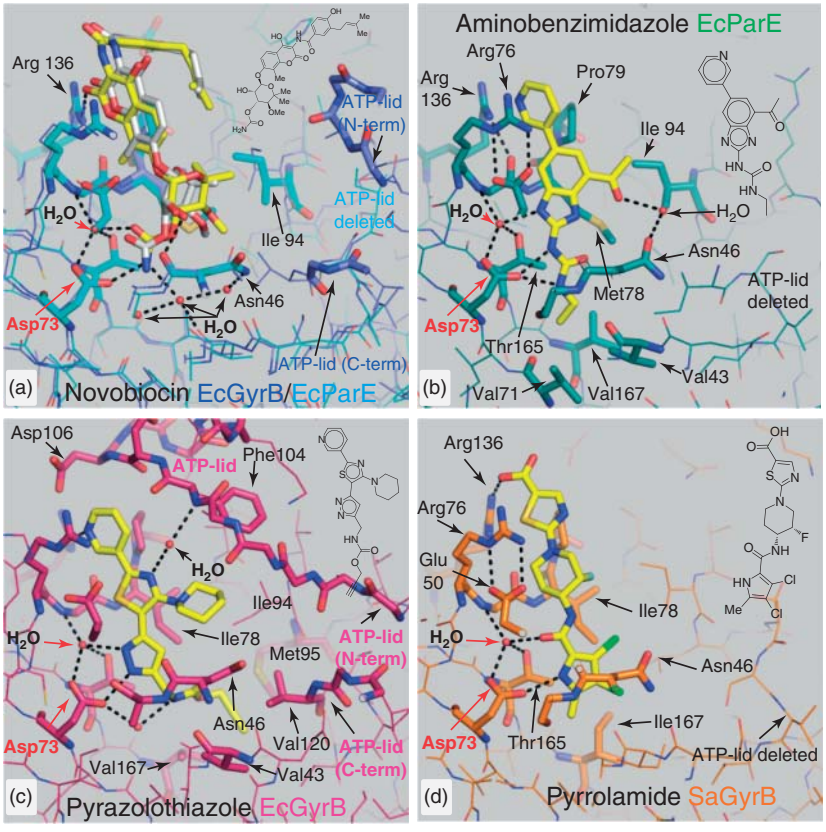
Figure 11.6 Comparison of AMPPNP (ATP analog) and novobiocin binding to ATPase domain of ParE or GyrB. (a, b) Two views of the 2.1 Å structure of the non-hydrolyzable ATP nucleotide analog, adenylyl-imidodiphosphate (AMPPNP) in complex with the *E. coli* ParE [87] (PDB code: 1S16). The binding of the nucleotide (magenta carbons) orders the ATP-lid region of GyrB/ParE (residues 99–119-shown as grey/blue/red mainchain sticks; *E. coli* GyrB numbering is used throughout figure for simplicity). The ATP-lid (residues 99–119) not only wraps around the ribose and phosphates of the nucleotide but also helps form the binding site for the N-terminal arm (residues 2–15) from the second subunit (black/dark gray) of the dimer (second subunit labels in orange fill). The adenine ring of the nucleotide makes hydrogen bonds to GyrB Tyr 5′, Asp73 and three waters (H-bonds indicated by dotted lines). (c) The original 2.7 Å structure of novobiocin in complex with the *E. coli* GyrB GHKL domain (dark blue) [84]; the ATP-lid region does not make contact with novobiocin. (d) The 2.0 Å crystal structure of novobiocin with *E. coli* ParE (light blue) [87] (PDB code: 1S14) superimposed on the AMPPNP complex (colored as in (b)). Novobiocin (yellow carbons) binds in the same pocket as Tyr5 and the adenine ring of the nucleotide. As a result, novobiocin blocks the binding of ATP and inhibits dimerization of the GyrB/ParE subunits (see Section 11.3.1.1 for details).

novobiocin, clorobiocin, and coumermycin. Novobiocin (Albamycin, Figure 11.2k), a sub μM inhibitor of gyrase, has excellent antistaphylococcal activity and was marketed by Upjohn for clinical use in the treatment of serious S. *aureus* infections [82], but had to be discontinued due to toxicity and efficacy concerns [83]. Its use has also been limited by weak gram-negative activity, poor oral absorption, and a propensity for the development of resistance [84].

The structure of novobiocin complexed to the ATP-binding domain of *E. coli* GyrB [84], showed that novobiocin bound in a pocket that overlapped with both the adenine ring of a non-hydrolyzable ATP analog, adenylyl-imidodiphosphate (AMPPNP), and Tyr 5′ of GyrB [85] (Figure 11.6). The residues forming this novobiocin-binding pocket (to the bottom and left in panels in Figure 11.6d), are in very similar positions in the novobiocin structure to those observed in the ATP-bound structure. However, a region of the ATP-binding pocket known as the *ATP-lid* (residues 99–119), which makes extensive contacts with the phosphates of the ATP, adopts a radically different conformation in the novobiocin structure and does not contact novobiocin. The binding of ATP induces a conformation of the ATP-lid, such that the ATP-lid forms part of the binding site for the N-terminal arm of the opposite subunit of the dimer, thus inducing dimer formation on ATP binding. Because the ATP-lid region is a conformationally flexible region, it can cause difficulties in crystallization; a number of crystal structures have been solved with ATP-lid deletion mutants – in which this loop has been deleted [86]. However, a recent pyrazolthiazole structure (Figure 11.7c, Section 11.3.2.4), showed a new conformation for the ATP-lid that was close to the inhibitor, suggesting some classes of ATPase inhibitors may make favorable interactions with the ATP-lid. However, for novobiocin, the ATP-lid did not contact the inhibitor (Figure 11.6c), and novobiocin bound similarly to an *E. coli* ParE ATP-lid deletion mutant to the *E. coli* GyrB intact structure (Figure 11.7a).

Figure 11.7 Four crystal structures of ATP-domain inhibitors in complex with ParE or GyrB. (a) Novobiocin. The 2.0 Å crystal structure of novobiocin (yellow carbons) with *E. coli* ParE (carbons light blue) (PDB code: 1S14) [87], superimposed with the 2.7 Å structure of novobiocin (white carbons) with *E. coli* GyrB (carbons dark blue) [84]. Residues within 3.8 Å of the inhibitor are shown in thicker ''stick'' representation; the backbone atoms of the ATP-lid residues are also shown as ''sticks.'' The *E. coli* ParE construct (light blue) has had the ATP-lid sequence deleted from the construct (residue 98–120 in *E. coli* GyrB numbering, Figure 11.7e). Note the deletion of the ATP-lid has not significantly altered the binding mode. Residue numbers used throughout the figure are *E. coli* GyrB numbers (as on sequence alignment). Red arrows indicate Asp73 and conserved water that form H-bonds with all four ATPase inhibitors (Figure 11.7a–d). (b) A 1.8 Å structure of an aminobenzimidazole (yellow) in complex with an ATP-lid deletion mutant of *E. coli* ParE (PDB code: 3fv5) [88]. (c) A 2.2 Å structure of a pyrazolothiazole (yellow) (PDB-code: 3G7E) [89] with the *E. coli* GyrB, residues coming within 3.8 Å of the inhibitor shown as magenta sticks (including side chains). Note contacts with ATP-lid region. (d) A 1.63 Å structure of a pyrrolamide in complex with an ATP-lid deletion mutant of *S. aureus* GyrB (PDB code 3ttz) [90]. (e) Sequence alignment highlighting amino acid residues contacting the four different classes of ATPase-domain inhibitors. Residues that contact all four inhibitors are colored purple and underlined. Three of the inhibitors (not novobiocin) extend toward the bottom of the figures (b–d), and make a varying number of additional contacts with residues highlighted in green on sequence alignment. Inhibitors that extend to the right-hand side of figure make additional contacts with residues in red on sequence alignment. The sequences shown are from: DNA gyrase (GYRB) and topo IV (PARE) from two gram-negative (Ec = *E. coli*, Ab = *A. baumannii*) and two gram-positive (Sa = *S. aureus*, Sp = *S. pneumoniae*) bacteria. The DNA gyrase sequence from *M. tuberculosis* (Mt) is also shown (Mt does not have a topo IV). Three eukaryotic sequences are also shown: human topo IIα and β (Hs = Homo sapiens) and yeast (Sc = *S. cerevisiae*) topo II.

A key interaction of novobiocin is with Asp73 and an adjacent water molecule located in the gyrase ATP-binding site (Figure 11.7a) – this interaction appears to be common to all ATP-competitive inhibitors (Figure 11.7) [14, 91]. Mutations in gram-positive and gram-negative bacteria that confer resistance to aminocoumarins map to the N-terminal domain of GyrB, which contains the site of ATP hydrolysis [14]. The most commonly mutated residue responsible for novobiocin resistance is R136

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          43  46  50    73  76  79    95     102 106  120  136  165
EcGYRB    VVDNAIDE  VQDDGRGIPTG  IMTVLHAGGKFDD  GVS  QRE  GTMVR
AbGYRB    VVDNAIDE  VSDNGRGIPTD  ILTILHAGGKFDD  GVS  YRA  GTTVR
SaGYRB    IVDNSIDE  VTDNGRGIPVD  ILTVLHAGGKFGG  GSS  HRN  GTVIR
SpGYRB    IVDNSIDE  VVDDGRGIPVD  VFTVLHAGGKFGG  GSS  HKN  GTTVH
MtGYRB    VVDNAVDE  VADDGRGIPVA  VMTQLHAGGKFDS  GVS  KRD  GSTVR
SaPARE    IVDNSVDE  IEDNGRGMPTG  IFTVLHAGGKFGQ  GAS  HRD  GTKVT
SpPARE    IVDNAVDE  VQDHGRGMPTG  IFTILHAGGKFGQ  GSS  TRD  GTKVT
AbPARE    VIDNAVDE  VEDNGRGMPVD  ILTKLHAGGKFST  GIS  QRQ  GTTVR
EcPARE    VIDNSVDE  VIDDGRGMPVD  ILCRLHAGGKFSN  GIS  RRD  GTSVH
HsTOP2A   ILVNAADN  IWNNGKGIPVV  IFGQLLTSSNYDD  GAK  ASR  YTCIT
HsTOP2B   ILVNAADN  IWNNGKGIPVV  IFGQLLTSSNYDD  GAK  ACK  YTCIT
ScTopII   ILVNAADN  VKNDGKGIPIE  IFGHLLTSSNYDD  GAK  ADL  YTKVT
(e)                                  99 ATP-LID 119
```

(*E. coli* GyrB numbering) and this key amino acid residue forms a hydrogen bond with the carbonyl oxygen of the aminocoumarin (Figure 11.7a) [92]. Novobiocin also binds with the ParE subunit of topo IV (Figure 11.7a) at a site similar to that found in its GyrB structure, but its activity against the topo IV enzyme is up to 12-fold less compared to gyrase, largely due to Ile78 in GyrB being a methionine in ParE (Figure 11.7b,c) [87, 93].

Recent developments regarding aminocoumarin inhibitors have focused on improving the activity of clorobiocin, which is about 10- and 70-fold more potent than novobiocin against DNA gyrase and topo IV respectively [94]. Several hybrids of clorobiocin have been produced by genetic engineering of a producer strain [95], but no analogs showed antibacterial activity improvements over clorobiocin itself [96].

11.3.1.2 **Cyclothialidines**

Cyclothialidines, isolated from *Streptomyces filipinesis*, are a heavily studied class of ATPase inhibitors, which were independently discovered in DNA gyrase supercoiling screens of natural products by Roche [97] and Glaxo researchers [98]. GR122222X (Figure 11.2l) differs by an alanine at the N-terminal amino acid compared to a serine in cyclothialidine. Cyclothialidines are competitive ATPase inhibitors, with antigyrase potency similar to novobiocin, that bind at distinct but overlapping sites in GyrB [84], showing only very low-level cross-resistance to novobiocin [98]. Comparison of the GyrB crystal structures of cyclothialidine with that of novobiocin showed that the phenolic groups of cyclothialidine and the novobiose sugar of novobiocin are involved in a common hydrogen bond network with D73 of GyrB and critical water molecules [84, 91]. Development of the cyclothialidine class of inhibitors has been limited by poor *in vivo* efficacy owing to extensive and rapid glucoronidation of the phenol moiety, which is essential for GyrB binding [14, 91].

Continued efforts to translate the potent enzyme activity of cyclothialidines into *in vivo* efficacy led to the discovery of several novel subclasses including phenolic [91, 99] and aminothiazole [100] DNA gyrase inhibitors, and bicyclic dilactone derivatives that introduced broad gram-positive antibacterial activity, and importantly improved *in vivo* efficacy [101]. Despite much structural design efforts, to date, no cyclothialidine class of inhibitors has advanced to the clinic.

11.3.1.3 **Kibdelomycin and Amycolamicin**

In an elegant chemical genomics study, Merck recently described the discovery of the natural product kibdelomycins as a completely novel class of GyrB inhibitors (Figure 11.2m), identified using a target-based, whole-cell screening approach and fitness testing [102]. Screening of a panel of 245 inducible antisense RNA strains of essential gene targets in *S. aureus* against crude natural product extracts resulted in specific sensitization of a kibdelomycin-containing fraction to the GyrB/GyrA and ParE/ParC antisense strains and depletion of these strains in the pool, suggesting the active compounds are bacterial topoisomerase inhibitors. Isolation and structural elucidation of the fermentation extract identified kibdelomycin as the active molecule. Macromolecular biosynthesis pathway analysis showed inhibition of DNA synthesis consistent with inhibition of bacterial topoisomerases [102].

Kibdelomycin has an MIC of 2 μg ml^{-1} against *S. aureus* but weak *E. coli* antibacterial activity owing to reduced permeability and/or efflux. Similar to novobiocin, kibdelomycin inhibited the ATPase activities of DNA gyrase and topo IV, and is more potent against DNA gyrase than topo IV with IC_{50} values of 11 and 900 nM, respectively, in *E. coli* gyrase and topo IV ATPase assays. Kibdelomycin did not show cross-resistance to a novobiocin-resistant *S. aureus* GyrB D89G mutant (GyrB G81 in *E. coli*, Figure 11.7e) or a ciprofloxacin-resistant mutant, while it displayed fourfold cross-resistance to a high-level coumermycin-resistant *S. aureus* mutant containing GyrB Q136E, I175T, and L455I mutations, suggesting that kibdelomycin has a unique binding mode. The frequency of spontaneous resistance of kibdelomycin was low at $<5 \times 10^{-10}$, significantly lower than novobiocin, which is reported to be 10^{-8} [102]. Hence, kibdelomycin is a novel class of ATPase inhibitor of DNA gyrase and topo IV with an apparently new binding mode although its precise binding pocket remains to be elucidated.

Independently, amycolamicin, belonging to the same structural class as kibdelomycin, was discovered by screening and isolation of natural products from actinomycetes [103, 104]. Amycolamicin possessed broad-spectrum gram-positive activity against clinically relevant bacteria including MRSA. Amycolamicin, as is the case with kibdelomycin, is more potent against gyrase than topo IV, and has good selectivity over human topo II [104]. Amycolamicin demonstrated good therapeutic efficacy against *S. aureus* in the murine septicemia model [104]. Understanding the structural basis of the antibacterial mode of action of amycolamicin and kibdelomycin may help lead to novel antibiotics derived from these novel natural products.

11.3.2
Recent GyrB and Dual-Targeting GyrB/ParE ATPase Inhibitors

An inhibitor with balanced, dual targeting of the GyrB/ParE ATPase subunits is highly attractive in reducing the frequency of development of drug resistance, as the likelihood of two simultaneous mutations occurring in two essential targets is low [87]. Aided by the availability of crystal structures of GyrB and ParE, many different classes of inhibitors with some evidence of dual mechanism have been published by various groups. Among them are the benzimidazoles by Vertex [88, 93, 105]; indazoles by Roche [91], Dainippon [106], and Quorex [14]; pyrazoles by Dainippon [107]; imidazopyridines by Pfizer [108]; triazolopyridines by Evotec/Prolysis [109]; and pyrrolopyrimidines and pyrimidoindoles by Trius [110, 111] as well as related structures disclosed in a number of patents (including benzothiazoles, thiazolopyridines, imidizaopyridazoles, and pyridines by AstraZeneca, Biota, and Ranbaxy – recently reviewed in [112]). Virtually all of these GyrB/ParE inhibitors seem to target gram-positive pathogens, and disappointingly none have thus far progressed for clinical use. Here, we discuss recent studies on novel dual-targeting ATPase inhibitors (Sections 11.3.2.1–11.3.2.3), the latest reports on novel GyrB inhibitors (Sections 11.3.2.4 and 11.3.2.5), and finally the prospects for the successful clinical progression of some of these new classes of ATPase inhibitors (Section 11.3.2.6).

11.3.2.1 Aminobenzimidazole Ureas

Aminobenzimidazole ureas were first identified as a novel class of dual GyrB/ParE ATPase inhibitors by Vertex following a GyrB HTS and structure-guided design [88, 93, 105]. A representative lead compound, VRT-752586 (Figure 11.2n) showed potent antibacterial activity against key multidrug-resistant gram-positive pathogens (MICs below 1 $\mu g\,ml^{-1}$ against quinolone-resistant isolates) and respiratory gram-negative pathogens. Inhibitors exhibited bactericidal activity [93] and showed promising *in vivo* efficacy in *S. aureus* and *S. pneumoniae* animal infection models [88]. VRT-752586 is a highly potent inhibitor of both DNA gyrase and topo IV enzymes, with respective K_i values of <4 and 23 nM in *E. coli* gyrase and topo IV ATPase assays [88], and gave a low frequency of spontaneous resistance such as $<5.7 \times 10^{-10}$ in *S. aureus* in accordance with its balanced dual-targeting properties [93]. Crystallographic studies identified T165, R136, and D73 of GyrB (*E. coli* numbering) as key residues that interact with the aminobenzimidazole ureas (Figure 11.7b) [88]. GyrB T165, a VRT-75286 resistance-conferring mutation found in all four bacteria studied, is a highly conserved residue and implicated in novobiocin resistance, consistent with VRT-75286 interacting with GyrB at a site that overlaps with the binding site of novobiocin (Figure 11.7a,b) [105]. Aminobenzimidazole GyrB inhibitors also showed antibacterial activity against drug-resistant mycobacteria and significant *in vivo* efficacy in a murine tuberculosis lung model [93, 113], suggesting early promise for TB therapy.

Despite much structure-guided efforts around Vertex's aminobenzimidazoles, a first in a novel class of potent dual ATPase inhibitors, this series of molecules has not so far advanced to the clinic. This lack of clinical success may have been attributed to the reported 16-fold increase in MIC observed in the presence of 50% human serum, suggesting high protein binding [93], while poor compound solubility may also have hindered their progression. The leading work by Vertex attracted renewed efforts around finding novel inhibitors targeting the ATP-binding domain. For example, Biota recently disclosed [112] a structurally related class of benzothiazole ureas, with antistaphylococcal bactericidal activity and efficacy in an *S. aureus* septicemia mouse model, that had been optimized to improve solubility and pharmacokinetic druglike properties. Additional ATPase domain inhibitors based around the benzimidazole structure are also discussed in the next sections.

11.3.2.2 Imidazopyridines and Triazolopyridines

Molecular docking studies around the benzimidazole scaffold by Prolysis (now Biota) and Evotec, led to discovery of triazolopyridine inhibitors that target the ATPase of both DNA gyrase and topo IV [109]. Although there was a significantly higher preference for inhibition of GyrB over ParE, an exemplar molecule exhibited a low resistance frequency of $<1.8 \times 10^{-9}$ in *S. aureus* [109] consistent with a dual mechanism of inhibition. The triazolopyridine inhibitors achieved single-digit MICs against key gram-positive pathogens including MRSA but lacked wild-type *E. coli* antibacterial activity likely due to efflux or permeability barrier [109]. Around the same time, Pfizer reported a novel program of pyrimidinyl-imidazo-pyridine

inhibitors (Figure 11.2o) that targeted both GyrB/ParE ATP-binding domains [108]. Several compounds were shown to be effective against fluoroquinolone-resistant *S. pneumoniae* and MRSA strains *in vitro* and, furthermore, demonstrated promising efficacy in mouse *S. pyogenes* sepsis and *S. pneumoniae* lung infection models following oral dosing at $<50\ mg\ kg^{-1}$. No further information on the advancement of this class of molecules is given.

11.3.2.3 Pyrrolopyrimidines and Pyrimidoindoles

To date, the development of GyrB/ParE inhibitors has been primarily focused against gram-positive bacteria, but there remains an urgent demand for novel agents to combat the rise in life-threatening infections caused by gram-negative pathogens. Toward this need, Trius reported the discovery of a novel series of pyrrolopyrimidine (Figure 11.2p) and pyrimidoindole nanomolar inhibitors of GyrB/ParE enzymes by fragment-based screening and structural optimization [110, 111]. These investigational antimicrobials showed impressive broad-spectrum antibacterial activity against key gram-positive (*S. aureus* MIC $\leq 0.03\ \mu g\ ml^{-1}$) and gram-negative pathogens (MICs of an exemplar pyrimidoindole of 0.5, 1, and 2 $\mu g\ ml^{-1}$ against wild-type *Acinetobacter baumannii*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*) [110] as well as activity against all class A biothreat organisms [114]. The frequency of spontaneous mutation in *E. coli* was extremely low at $<3.8 \times 10^{-11}$ [115] in accordance with potent and balanced dual targeting of GyrB and ParE enzymes by these inhibitors (K_is of <0.05 and <0.2 nM, respectively, in *E. coli* ATPase enzyme assays) [110]. Mutants selected by serial passage in an *E. coli tolC* strain showed up to 16-fold elevated MICs to pyrimidoindoles that was associated with mutations in GyrB V120F, the upstream region of *gyrB* as well as nontarget mutations [115]. *In vivo* efficacy has also been demonstrated in *E. coli* and *S. pneumoniae* murine infection models [116]. Initial preclinical studies around this novel class of dual GyrB/ParE inhibitors are encouraging although further safety data are needed [110]. Unlike the other ATPase inhibitor class, the pyrrolopyrimidines and pyrimidoindoles are particularly active against gram-negative pathogens.

11.3.2.4 Pyrazolthiazoles

To identify novel chemotypes for finding new classes of GyrB inhibitors, an HTS was run by Vertex against the GyrB subunit that led to the discovery of pyrazolthiazoles as novel and potent inhibitors of GyrB (Figure 11.2q) [89]. Structural studies showed that the pyrazole core of the inhibitor makes hydrogen bond to GyrB D73 and a highly conserved water (Figure 11.7c), while the propynyl group extends into a pocket lined by three hydrophobic residues (V43, M95, and V167). Unusually, in the *E. coli* GyrB crystal structure, the ATP-lid (residues 99–119) adopted a previously unobserved conformation in which it made a number of van der Waals contacts with the inhibitor (Figure 11.7c). Analogs displayed potent enzyme inhibition of GyrB and antibacterial activity against *S. aureus* and *S. pneumoniae*, but weak MICs against *E. coli* due to active efflux. Of interest, the pyrazolthiazole classes are selective enzyme inhibitors of *E. coli* GyrB over *S. aureus* GyrB, which was

explained by differences in their binding sites [89], and may suggest opportunities for their exploitation as gram-negative agents.

11.3.2.5 Pyrrolamides

Most recently, a fragment-based NMR screening approach by AstraZeneca led to the identification of the novel class of pyrrolamide inhibitors of DNA gyrase B [90, 117]. Pyrrolamides (Figure 11.2r) are effective against gram-positive pathogens with good antibacterial activity against quinolone-resistant *S. aureus* (MIC of 2 $\mu g\,ml^{-1}$), but have limited spectrum against gram-negative pathogens owing to efflux. *In vivo* efficacy was demonstrated in an *S. pneumoniae* mouse model of pneumonia [90]. Pyrrolamides are bactericidal, demonstrating that an ATP-binding competitor can be lethal [117]. Spontaneous-resistant mutants to pyrrolamide in *S. aureus* mapped to GyrB R144 and T173 (equivalent to R136 and T165 in *E. coli*) and these same two amino acids make key contacts with the compound in the GyrB co-crystal structure with the ATP-binding domain (Figure 11.7d) [117]. The frequency of spontaneous resistance was 2×10^{-9} in *S. aureus*, suggesting that rapid emergence of pyrrolamide resistance is unlikely. Pyrrolamides are potent nM inhibitors of the ATPase activity of *S. aureus* DNA gyrase [90], but did not significantly inhibit ParE of topo IV [117].

11.3.2.6 Clinical Progression of ATPase Inhibitors

Despite huge efforts over many years by many companies, and the availability of GyrB and ParE crystal structures for structure-guided approaches, to the authors' knowledge, novobiocin remains the only ATPase inhibitor to have reached advanced clinical testing. Recently, AstraZeneca reported a gyrase B inhibitor, AZD5099, of undisclosed chemical structure, that is in phase 1 for treatment of serious infections; however, it is being discontinued for safety and efficacy reasons [118]. The fact that so few advanced clinical compounds that target the catalytic ATPase domain have emerged, illustrates the challenges of antibacterial drug discovery. The attractions of the low frequency of spontaneous resistance by targeting both GyrB and ParE ATP-binding sites, the potential to overcome resistance to marketed quinolones, and the opportunity of finding selective ATPase inhibitors with a low toxicological profile, warrants further research efforts. Novel scaffolds are being found, with one example of a new class of dual-targeting inhibitors being the pyrimidoindoles that show promising gram-negative antibacterial activity [110].

11.4 Simocyclinones, Gyramides, and Other Miscellaneous Inhibitors

11.4.1 Simocyclinone D8

Simocyclinone D8 (SD8; Figure 11.2s) is an aminocoumarin polyketide produced by *Streptomyces antibioticus* with a novel mechanism of action by binding to the

N-terminal GyrA catalytic domain (Figure 11.1, panel 11.4), and preventing gyrase from binding to DNA [119, 120]. Although it has an aminocoumarin moiety similar to novobiocin, it does not inhibit the GyrB ATPase activity [119], neither does it stimulate DNA cleavage formation [121], differentiating it from novobiocin- and quinolone-type mechanisms. SD8 is a potent inhibitor of the supercoiling activity of DNA gyrase with IC_{50} of 0.4 and 1.4 μM for *E. coli* and *S. aureus* gyrase enzymes, respectively, and is much less active against topo IV of *E. coli* (> 650-fold less) and *S. aureus* (10-fold less) [122]. Confirming the molecular target of SD8 as DNA gyrase, mutants resistant to SD8 in *E. coli* mapped to *gyrA*, and these mutations (V44G, H45Y, G81S, and D87Y) are located close (Figure 11.8) to the bound SD8 molecule in the crystal structure [119]. The binding site of SD8 is close to the quinolone-binding site, consistent with some level of cross-resistance between SD8 and ciprofloxacin mutants [119, 123]. Intriguingly, recent studies suggest a possible binding site of SD8 at the C-terminal domain of GyrB that is distinct from the known N-terminal domain of GyrA [124]. The molecular weight of SD8 (associated with cooperative occupancy of two distinct binding sites) could make it challenging to encompass gram-negative pathogens [27] into the antibacterial spectrum of this structural class. However, SD8 has exhibited activity against some clinical gram-negative isolates that have enhanced drug uptake in comparison to laboratory strains, indicating some potential for combating gram negatives [123]. Exploitation of the novel simocyclinone-binding pockets, supported by structure-guided approaches, may potentially lead to more active analogs and the discovery of novel gyrase inhibitors acting by a similar mechanism.

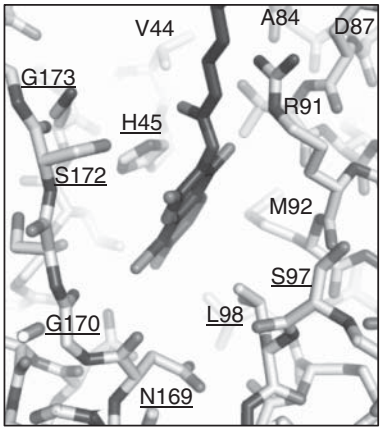

Figure 11.8 The aminocoumarin moiety of simocyclinone D8 (black carbons) binds in a pocket in *E. coli* GyrA (white/gray carbons) [119], which is surrounded by a number of residues (labeled and underlined) which, when mutated, confer resistance to gyramide A [125], suggesting gyramide A may also bind at this pocket. In complexes with DNA, residues equivalent to H45 and R91 interact with a phosphate from the DNA backbone, SD8 will block this interaction and hence DNA binding.

11.4.2 Gyramides

Gyramides are a recently discovered, novel class of benzylsulfonamidopyrrolidines [125] that inhibit DNA gyrase. Interestingly, the gyramides were discovered in a cell-based screen designed to identify small molecule inhibitors of cell division in *E. coli*, and produced extensive filamentous cells but did not significantly induce FtsZ GTPase or the SOS response [126]. Gyrase was identified as the molecular target by deep DNA sequencing of two gyramide-resistant mutants [125]. Gyramide C showed micromolar inhibition in the *E. coli* DNA gyrase supercoiling assay that tracked its antibacterial activity in an *E. coli*-efflux-deficient mutant, and displayed moderate activity against gram-positive bacteria. Mutants resistant to gyramide A all mapped to *gyrA* or *gyrB* (15 different mutations in *gyrA* and 1 in *gyrB*) [125]. Ciprofloxacin did not show elevated MIC to the gyramide-resistant mutants, suggesting different binding sites. About 50% of the gyramide mutations (GyrA H45Q or Y, L98Q or P, N169K, G170C, S172P, and G173C) clustered around the binding pocket for simocyclinone D8, perhaps suggesting similar binding sites (Figure 11.8). Unlike quinolones, but similar to simocyclinone, gyramides did not induce DNA cleavage. These data suggest that gyramides may function, similar to simocyclinones, as inhibitors of DNA binding; however, more experiments are needed to validate this hypothesis.

11.4.3 Other Miscellaneous Inhibitors

11.4.3.1 Pyrazoles

An interesting series of pyrazole inhibitors were discovered by Dainippon in a whole-cell screen for specific inhibitors of chromosome partitioning in *E. coli* [107, 127]. Mechanistic studies of the lead pyrazole inhibitor, ES-1273, revealed the inhibitor did not inhibit ATPase activity or cleave DNA but instead prevented DNA from binding to DNA gyrase, suggesting a novel mode of action. However, ES-1273 was poorly selective with respect to human topo II for development [128].

11.4.3.2 Quercetin Derivatives

The flavanoid quercetin has been shown to bind the DNA gyrase B inhibiting ATPase activity, and to interact with DNA [129]. Recently, from a structure-based GyrB docking effort, quercetin diacylglycoside analogs that are low micromolar dual gyrase/topo IV inhibitors with potent antibacterial activity against MRSA strains were described [130]. However, their precise mode of action and selectivity over eukaryotic topo II were not reported.

11.4.3.3 Macromolecular Inhibitors of DNA Binding

Naturally occurring endogenous proteins inhibit type II topoisomerases by sequestering DNA gyrase away from DNA, including YacG [131], GyrI [132], MurI [133], and Qnr/MfpA [134, 135]. Understanding the unique binding modes of diverse

macromolecular inhibitors may also help rational design of new small molecule analogs as antibacterial agents against DNA gyrase.

11.5 Conclusions and Perspectives

Bacterial DNA gyrase and topo IV inhibitors represent an impressive structural and mechanistic diversity. Many of these learnings originated from determining the mode of action of compounds that had initially been discovered by simple antibacterial testing (e.g., quinolones, NBTIs, QPT-1, simocyclinone, clerocidin, and novobiocin). Many essential bacterial gene products are devoid of known inhibitors, and ''building in'' antibacterial activity into novel classes of inhibitors can be challenging [5]. This contrasts with the tractability and productivity of type II topoisomerases as targets for antibacterial leads. Targeted screening for leads has broadened the repertoire of known inhibitors of gyrase and topo IV, and targeted whole-cell methods will also likely continue to contribute to future discoveries, as recently exemplified by kibdelomycins and gyramides.

However, despite substantial pharmaceutical industry effort, DNA gyrase and topo IV have yet to realize their potential for new classes of drugs, as the fluoroquinolones are the ''only'' class of marketed antibacterials acting on gyrase and topo IV. This does not necessarily highlight a limitation of the druggability of this target class, but rather may be a microcosm for the challenges and attrition in antibacterial drug discovery and development in general, and for novel inhibitor classes especially. Generally, the more novel the drug and target the greater opportunity for transformational drug development, but also the greater the possibility for myriad developability ''surprises'' that can derail a drug's development [136]. While several marketed fluoroquinolones have been withdrawn (e.g., gatifloxacin, grepafloxacin, tosufloxacin, and trovafloxacin) [25], illustrating the challenges even for established drug families, the fluoroquinolones remain a highly productive and promising class for exploration. This is exemplified by delafloxacin and JNJ-Q2, which are in phase 2, and show interesting potential for expanding the antibacterial spectrum of fluoroquinolones to MRSA. Furthermore, on the basis of structural, biochemical, or genetic data, compounds such as quinazoline-2,4-diones, HITZ's, and QPT-1 demonstrate the exploitability of the fluoroquinolone mechanism by diverse chemistries. Interaction with GyrB rather than GyrA may explain the ability of these compounds to evade target-mediated cross-resistance with fluoroquinolones, as shown by mutational data, and also shown structurally with the quinazoline-2,4-dione, PD 0305970.

The recent discovery of the structural basis of action of quinolones and NBTIs provides new learnings for the exploitation of DNA gyrase and topo IV, adding to the more established crystallography on the ATPase domain. Structure-based approaches can underpin the lead optimization of these inhibitor classes and help create new classes. The interfacial inhibition mechanisms of quinolones, NBTIs, and other inhibitor classes are complex systems for *de novo* design and molecular

modeling, as the environment is very dynamic, with different macromolecules moving within a complex molecular machine. Furthermore, the inhibition paradigm of quinolones and NBTIs, as well as many other topoisomerase inhibitors, involves stabilization of complexes, and not ''simple'' competitive inhibition [137]. However, as more structural data emerges for different inhibitor classes acting at the DNA cleavage-religation site, more opportunities for knowledge-based approaches are likely. This stands to provide a ''new twist'' to the exploitation of this important drug target, for the discovery of badly needed new antibacterial agents.

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12
Antibiotics Targeting Bacterial RNA Polymerase

Konstantin Brodolin

12.1
Introduction

Transcription, a central process in the expression of genetic information in the cell, is performed by multisubunit DNA-dependent-RNA polymerases. Bacterial ribonucleic acid polymerase (RNAP) is a complex molecular machine, composed of the catalytic core (five subunits $\alpha_2\beta\beta'\omega$) and one of the promoter-specific sigma (σ) subunits required for transcription initiation. The σ subunit confers to the holoenzyme an ability to recognize the -10 and -35 promoter consensus elements, to melt promoter DNA at the transcription start site, and to initiate RNA synthesis. The bacterial transcription cycle (Figure 12.1) comprises four steps: (i) assembly of the holoenzyme from σ and the core, (ii) promoter complex formation and *de novo* initiation of RNA synthesis, (iii) processive elongation of the nascent RNA chain, and (iv) termination. Transcription initiation starts from reversible binding of the RNAP to promoter DNA, leading to the formation of the ''closed complex'' (RP_c). RP_c isomerizes into the transcriptionally competent ''open complex'' (RP_o), in which ~13 bp of promoter DNA around the transcription start site is melted to form a transcription bubble. The antisense DNA strand of the bubble enters into the RNAP active site and serves as a template for the initiation of RNA synthesis. During the first stage of RNA synthesis, called *abortive initiation*, RNAP remains bound to promoter DNA and reiteratively synthesizes short RNAs that are released from the active site. When the length of the RNA extends beyond 10–11 nt, a productive elongation complex forms and RNAP escapes from the promoter. After the promoter escapes, the contacts between σ and the core become partially disrupted and the σ subunit dissociates from the elongation complex. During termination, RNAP releases mRNA, dissociates from the DNA template, and becomes available for the next initiation event (reviewed in [1]).

Several structures of RNAPs from the *Thermus* genus have been solved [2, 3]. The structure of RNAP resembles a crab claw, with the pincers (or jaws) formed by the mobile clamp domain (primarily the β' subunit) and β subunit lobes (Figure 12.2). The catalytic site, marked by the presence of a Mg^{2+} ion, is deeply buried in the cleft between the pincers. The structure comprises four channels: the ''main channel''

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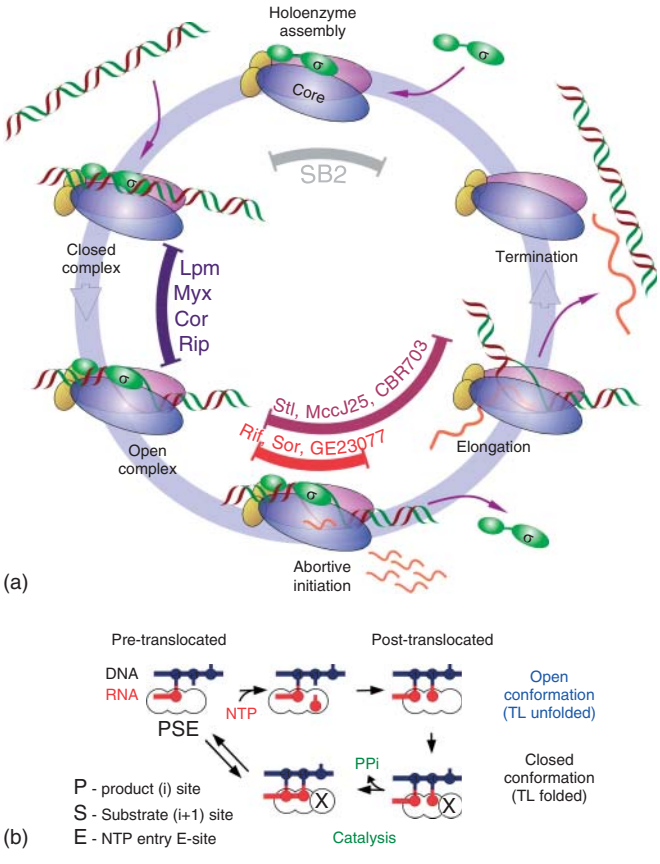

Figure 12.1 The transcription cycle of bacterial RNAP and transcriptional inhibitors. (a) A scheme showing the principal steps of transcription cycle: assembly of holoenzyme from core and σ subunit; promoter binding ("closed complex" formation); promoter melting ("open complex" formation); initiation of abortive RNA synthesis; elongation; and termination. The steps inhibited by the antibiotics are marked by the arcs. Lpm – lipiarmycin, Myx – myxopyronin, Cor – corallopyronin, Rip – ripostatin, Rif – rifampicin, Sor – sorangicin, Stl – streptolydigin, and MccJ25 – microcin J25. (b) A scheme showing the principal steps of nucleotide addition cycle (NAC) performed by the RNAP active center. TL – trigger loop.

that holds 8–9nt of the DNA/RNA hybrid, the dwDNA channel that holds the downstream part of DNA (promoter positions +1 to +15), the RNA-exit channel holding the nascent RNA chain, and the nucleoside triphosphate (NTP)-entry (secondary) channel believed to direct the NTP substrates to the catalytic site. The β′ clamp domain serves as a docking site for the σ subunit. The weakly conserved region 3.2 of σ forms an unfolded linker between the σ promoter recognition regions 2 and 4 and fills the RNA-exit channel in the holoenzyme. The linker must be ejected from the channel and replaced by the nascent RNA chain on the promoter escape. The clamp domain is linked to the core by the five switch regions: β′ switch

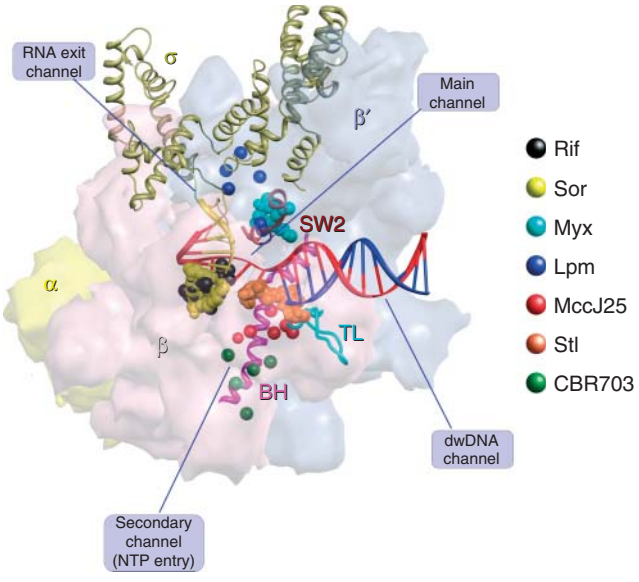

Figure 12.2 Localization of the transcriptional inhibitors binding sites on the structure of RNAP. Structural model of the *T. thermophilus* RNAP in complex with DNA fragment and RNA chain [3, 5]. RNAP is shown as a molecular surface with the β subunit colored in light pink, β′ subunit in light blue, and α subunit in yellow. The σ subunit is shown as ribbons colored in khaki. DNA is shown in red (template strand) and blue (nontemplate strand), the 10 nt RNA chain in yellow. The bridge helix (BH) in magenta, trigger-loop (TL) in cyan and switch-2 region (SW2) in brown are shown as ribbons. The antibiotics crystallized in complex with RNAP are shown as CPK: Rif in black, Sor in yellow, Myx in cyan, and Stl in orange. The mutations conferring resistance to Lpm (blue), CBR703 (green), and MccJ25 (red) are shown as spheres.

1, 2, and 5 and β switch 3 and 4. [2, 4]. Catalysis is performed by the two Mg^{2+} ions held by the triad of aspartate residues in the β′ subunit (*Escherichia coli* D460, D462, and D464). During the nucleotide addition cycle (NAC), the RNAP active center transfers a nucleotidyl moiety from the 5′-NTP in the substrate site ($i+1$-site) to the 3′-hydroxyl of the nascent RNA chain at the product site (i-site) (Figure 12.2). After phosphodiester bond formation, the 3′-end of RNA translocates from the $i+1$-site to the i-site and the $i+1$-site becomes available for binding to the next NTP. This translocation is driven by conformational cycling of the β′ subunit elements between ''trigger loop'' (TL, folded ↔ unfolded) and ''bridge helix'' (BH, bended ↔ straight).

Bacterial RNAP is a validated target for the development of highly specific antibacterial drugs because (i) it is an essential enzyme for cell survival, (ii) the basic structure of RNAPs is highly conserved between bacteria and less conserved between bacteria and eukaryotes, and (iii) the transcription regulatory mechanisms are different between eukaryotes and prokaryotes. A large number of antibacterial molecules, either natural or synthetic, bind to RNAP and inhibit different stages of the transcription cycle (Table 12.1) [6]. Among these molecules, rifampicin (Rif) and several of its analogs have reached clinical use and remain first-line antibiotics

Table 12.1 Main classes of transcription inhibitors.

	Compound	Development stage/ targeted pathogen	Source/nature	Spectrum of activity	Cross-resistance	Molecular target	Targeted process
Ansamycins	Rifampicin	Approved in 1968/ MTB	*Amycolatopsis mediterranei*/fermentation + semisynthesis	Gram positive, Gram negative	Sorangicin, streptolydigin	Main channel, *RpoB*	Extension of 2–3 nt. RNA chains
	Rifabutin	Approved in 1992/ MAC					
	Rifapentine	Approved in 1998/ MTB					
	Rifaximin	Approved in 2004/ enteropathogenic *E.coli*					
	Rifalazil	Phase II-III/MTB, *Chlamydia*					
Sorangicin		—	*Sorangium cellulosum*/ fermentation	Gram positive, Gram negative	Rifampicin		
Streptolydigin		—	*Streptomyces lydicus*/ fermentation	Gram positive	Rifampicin, microcin J25	dwDNA channel, *RpoB, RpoC*	RNA chain initiation and elongation
Microcin J25		—	*E. coli* AY25/fermentation	Gram negative	Capistruin	Secondary channel, *RpoB, RpoC*	
Capistruin		—	*Burkholderia thailandensis*/fermentation	Gram negative	ND	ND	
CBR703 series		—	HTS of chemical library/synthesis	Gram positive, Gram negative	ND	*RpoC, RpoB*	

Myxopyronin	—	*Myxococcus fulvus*/fermentation	Gram positive, Gram negative	Corallopyronin, ripostatin	Switch regions, *RpoB, RpoC*	Promoter complex formation
Corallopyronin	—	*Corallococcus coralloides*/fermentation	Gram positive, *Moraxella catarrhalis*	Myxopyronin, ripostatin		
Ripostatin	—	*Sorangium cellulosum*/fermentation	Gram positive, *Moraxella catarrhalis*	Myxopyronin, corallopyronin		
Lipiarmycin (fidaxomicin)	Approved in 2011/ *Clostridium difficile*	*Actinoplanes deccanensis*/fermentation	Gram positive, *Moraxella catarrhalis*	Myxopyronin		
GE23077	—	*Actinomadura* sp./ fermentation	*Moraxella catarrhalis*	ND	ND	RNA chain initiation
SB2 series	—	HTS of ChemBridge™ library/synthesis	Gram positive, Gram negative	ND	ND	Holoenzyme assembly
Ureidothiophene	—	HTS of chemical library/synthesis	*Staphylococcus*	ND	ND	ND

MTB, *Mycobacterium tuberculosis*; MAC, *Mycobacterium avium complex*; ND, not determined; HTS, high-throughput screening.

for the treatment of tuberculosis. Recently, a second RNAP-targeting antibiotic, lipiarmycin (Lpm), was approved for clinical use under the name Dificid (Optimer Pharmaceuticals, USA) for the treatment of *Clostridium difficile* infections. These drugs validate RNAP as a potent target for future drug development. Considering the mechanism of inhibition and target site (if known), the inhibitors of RNAP can be grouped into one of the four groups: (i) blocking nascent RNA extension, (ii) targeting RNAP active center, (iii) blocking promoter complex formation, and (iv) hindering σ–core interactions.

12.2
Antibiotics Blocking Nascent RNA Extension

12.2.1
Ansamycins (Rifamycins)

Ansamycins are a large family of antibiotics produced by *Actinomycetes* (reviewed in [7]). The name comes from the Latin word ''ansa,'' meaning – handle, from the basket-like chemical structure characterized by an aromatic moiety (planar naphtoquinone ring system) bridged at two nonadjacent positions by an aliphatic chain. Streptovarycine was the first described transcriptional inhibitor of the ansamycins family; the compound was isolated from *Streptomyces spectabilis* and was proposed as an antituberculosis drug [8]. However, rifamycines, isolated by Sensi and coworkers in 1959 [9] from *Amycolatopsis mediterranei* (previously known as *Streptomyces mediterranei* or *Nocardia mediterranei*), appeared to be more potent antibacterials. The original metabolite, rifamycin B, was moderately active and was chemically modified to produce more efficient derivatives (Figure 12.3). The most successful one, Rif (MW ~ 823 Da), was introduced in 1968 and still remains the first-line drug for the treatment of tuberculosis. Rif is the most potent inhibitor of RNAP with a K_i value of ~1 nM (*E. coli* RNAP). Three analogs of Rif; rifabutin, rifaximin, and rifapentine, produced through chemical modification of Rif at positions 3 and 4 ((Figure 12.3), reviewed in [10]), were approved for clinical use for the treatment of a broad range of infectious diseases. Another efficient derivative, rifalazil or KRM-1648 (ActivBiotics Pharma), displayed 10- to 100-fold better activity than Rif and is currently under development for the treatment of tuberculosis and *Chlamydia trachomatis* infections [10]. Rifamycines are broad-spectrum antimicrobials and exhibit the highest level of activity against gram-positive bacteria (MIC of $<0.1\,\mu g\,ml^{-1}$), as well as good activity against *Staphylococcus epidermis* biofilms [11] and, to a lesser extent, against gram-negative strains. Interestingly, Rif is also active against bacterial-type RNAP from apicoplast, the plastid of the malaria parasite *Plasmodium falciparum* [12]. Because Rif causes a high frequency (10^{-7} to 10^{-8}) of spontaneous Rif^R mutations that are rapidly selected during treatment [13], the drug should preferentially be used in combinational chemotherapy.

Rifamycines inhibit the synthesis of the RNA chains longer than 2–3 nts (depending on the presence of the 5′ phosphate in the priming NTP) and are

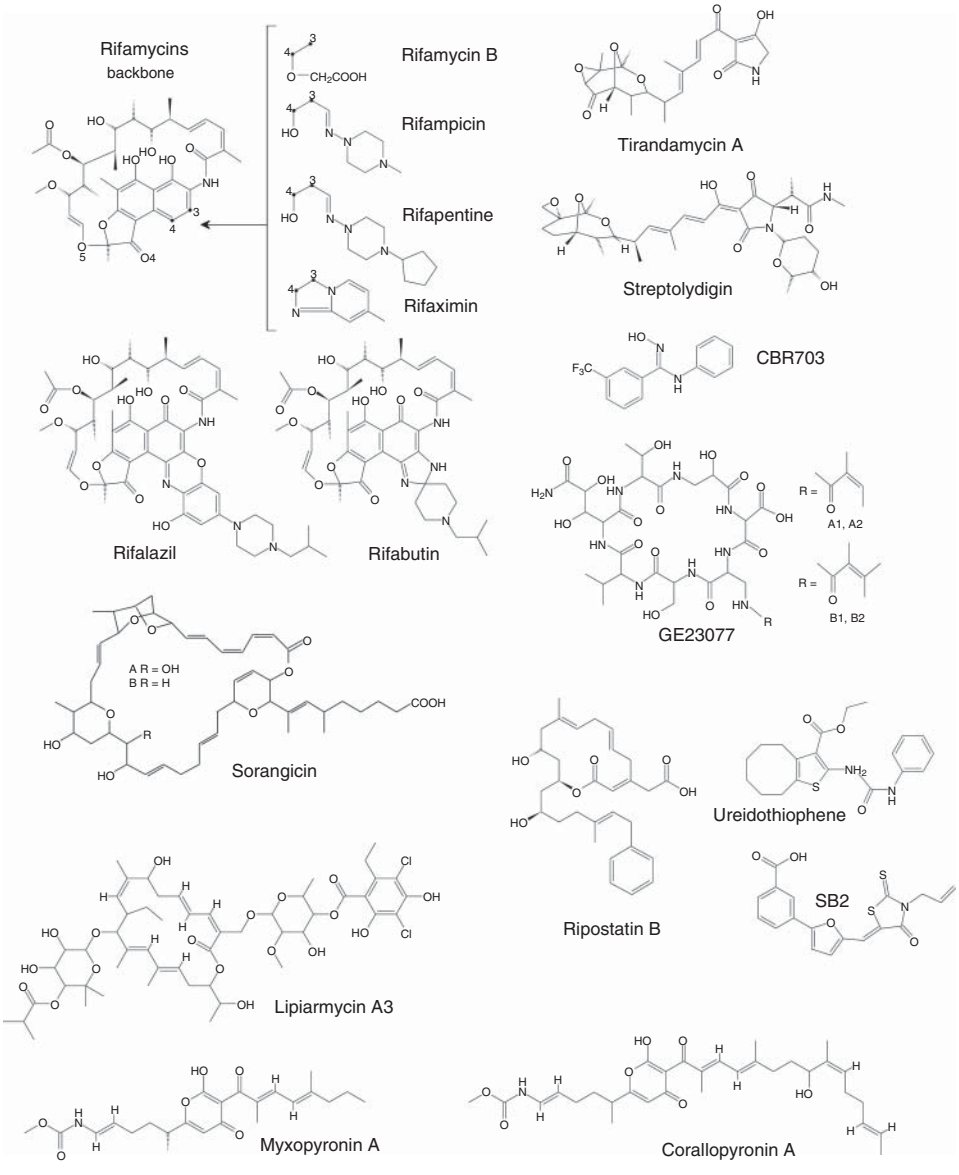

Figure 12.3 Chemical structures of transcription inhibitors.

not active on RNAP in the elongation complex. The structures of the *Thermus aquaticus* RNAP core with Rif at 3.3 Å resolution [14] and the *Thermus thermophilus* RNAP holoenzyme in complex with rifapentin and rifabutin at 2.5 Å resolution were solved [15]. The structures show that Rif binds exclusively to the RNAP β subunit within the main RNA/DNA hybrid channel, 12 Å away from the active

site. The binding site of the molecule (Rif pocket) comprises 16 amino acids, and mutations conferring resistance are located at 24 positions in the *rpo*B gene coding for the RNAP β subunit [16]. Resistance to Rif occurs either due to mutation of the residues forming contacts with the antibiotic or the surrounding residues that may affect the conformation of the binding site [14]. All Rif^R mutations are clustered within the ''Rif pocket'' in the central region of the β subunit within cluster I (amino acids 507–534, *E. coli* numbering is used in the text if not indicated otherwise), cluster II (amino acids 563–572), and cluster III (684–690) and near the N-terminus of the β subunit (residues 143, 146) [16]. On the basis of the *T. aquaticus* core RNAP structure, the ''steric occlusion'' model was proposed as the inhibition mechanism [14, 17]. According to the model, the binding of Rif to the main channel sterically blocks extension of the nascent RNA chain beyond 2–3 nts. A different ''allosteric'' model of inhibition was derived from structures of the rifamycin–RNAP holoenzyme complex lacking the catalytic Mg^{2+} ion. It was proposed that rifamycin binding induces an allosteric signal that propagates to the active site and disfavors Mg^{2+} binding, in turn slowing down catalysis and destabilizing the retention of short RNAs in the active site [15]. The two models are not mutually exclusive, and both mechanisms may contribute to inhibition. An important finding drawn from the rifamycin-holoenzyme structure was that residue D513 of the σ subunit region 3.2 contacts the O4 and O5 groups of the rifamycin ansa ring. Deletion in the σ region 3.2 renders RNAP resistant to rifabutin, suggesting that σ is implicated in inhibition [15]. Altogether, these data lead to the prediction that σ factors can modulate the sensitivity of RNAP to Rif. Indeed, it was demonstrated that *E. coli* RNAP containing the heat-shock σ^{32} is less sensitive to Rif than RNAP containing the housekeeping σ^{70} [18]. Bacteria have developed numerous mechanisms of resistance to rifamycins. The most frequent are mutations in *rpo*B, inactivation of Rif, or altered membrane permeability [16]. The producer strains are naturally resistant to Rif and streptovarycin owing to substitutions in the *rpo*B [19].

12.2.2 Sorangicin

Sorangicin (Sor, MW ~806 Da) is a macrolide polyester antibiotic produced in two structural variants (A and B) by myxobacterium *Sorangium cellulosum* [20]. Sor is a broad-range antibiotic that exhibits its highest level activity against gram-positive microorganisms including *Mycobacteria* (MIC of 0.01–0.1 $\mu g\,ml^{-1}$) and weaker activity against gram-negative bacteria (MIC of 3–30 $\mu g\,ml^{-1}$) [21]. The complete chemical synthesis of Sor has been described [22], and ~100 derivatives have been synthesized, some with improved activity against *Staphylococcus aureus* [23]. The antibiotic specifically inhibits transcription initiation by RNAP but not by the eukaryotic RNAP [21]. In contrast to Rif, which is weakly active against *Thermus* RNAP (IC_{50} of >100 μM), Sor efficiently inhibits both *E. coli* RNAP and *T. aquaticus* RNAP with IC_{50} values between 0.1 and 1 μM [24].

The structure of the *T. aquaticus* RNAP core in complex with Sor was resolved to 3.2 Å [24], revealing almost complete overlap between the Sor- and Rif-binding sites. Because Sor binds to the same binding pocket on the β subunit as Rif, the mechanism of inhibition is essentially the same as for Rif. Sor blocks the synthesis of the second phosphodiester bond as a result of the steric clashing with nascent RNA transcripts longer than 2–3 nts (the length depends on the presence of the phosphate group on the 5′ NTP) [24].

Owing to overlap in binding sites, there is substantial but not complete cross-resistance between Sor and Rif. The 12 residues of the β subunit that interact with Rif also interact with Sor. Three types of *rpo*B mutations in the Sor/Rif-binding pocket have been defined. The class I mutations, leading to both Rif- and Sor-resistance (Rif^R/Sor^R), affect the interactions critical for antibiotic binding. The class II mutations, conferring Rif-resistance and sensitivity to Sor (Rif^R/Sor^S), introduce distortions in the antibiotic-binding site that abolish Rif binding but have little or no effect on Sor binding. The class III mutants, conferring resistance to Sor and sensitive to Rif (Rif^S/Sor^R), affect β subunit residues 513 and 574, which form critical hydrogen bonds with Rif and Sor. Class III mutations do not significantly affect Rif binding but are incompatible with Sor binding. The appearance of class II mutations suggests that the Sor structure is flexible and can accommodate different conformations of the binding site. The conformational flexibility of Sor gives this compound an advantage over Rif and makes it a promising candidate for the treatment of Rif-resistant pathogens. For example, substitution of *Mycobacterium tuberculosis* Ser^{450} (*E. coli* Ser^{531}), which is found in 41% of clinical Rif^R isolates, results in Class II mutations. [24]. However, spontaneous Sor^R mutations can be easily selected with frequencies similar to Rif and fall into the Classes I and II [25].

12.3 Antibiotics Targeting RNAP Active Center

12.3.1 Streptolydigin and Other Acyl-Tetramic Acid Family Antibiotics

Streptolydigin (Stl, MW ~ 600 Da), also known as *portamycin*, was isolated from the actinomycete *Streptomyces lydicus* in 1955 and is a member of the 3-acyltetramic acid family of compounds with broad biological activities (antibacterial, anticancer, and antiviral) [26, 27]. Stl is active against the gram-positive bacteria *Staphylococcus, Streptococcus, Bacillus*, and *Corynebacterium* (MIC of 0.19–3.12 $\mu g\,ml^{-1}$) [11, 26, 28], whereas *E. coli* and other gram-negative bacteria are resistant to Stl owing to its low membrane permeability. Recently, a 29 *slg*-gene cluster responsible for the biosynthesis of Stl in *S. lydicus* was characterized [29]. In addition, the chemical synthesis of Stl and some of its analogs has been described [30], providing tools for the optimization and development of tetramic acid antimicrobial and anticancer reagents.

Stl specifically inhibits transcription initiation and elongation reactions carried out by RNAP with a K_i value of ~18 μM [28, 31], but is not active against eukaryotic RNAPs [32]. Biochemical studies showed that Stl decreases the rate of NTP addition, inhibits pyrophosphorolysis (PP_i-driven hydrolysis), and mRNA cleavage mediated by the elongation factor GreA, but does not affect substrate binding and phosphodiester bond formation [33]. Structures of the *T. thermophilus* RNAP holoenzyme-Stl complex [31, 33] and the *t*ranscription *e*longation *c*omplex (TEC) with Stl [5 were solved, revealing that Stl binds within the RNAP dwDNA channel 20 Å away from the active site. In agreement with the genetic data, the Stl-binding site is formed by β subunit residues 543–545 and 570–571 (Stl pocket) [34] and β′ subunit residues 788–798 (BH), 926–940 (TL), 1136–1139, and 1246 [31]. On the basis of the structural and biochemical data, it was suggested that Stl impedes NAC by interfering with conformational cycling of the RNAP BH and TL domains [5, 31, 33]. Binding of Stl stabilizes the BH in the straight conformation [31], leads to a 15 Å displacement of the TL, and freezes the catalytic site in the inactive ''open'' conformation [5, 33].

Spontaneous mutations leading to the Stl^R phenotype map to the β′ and β subunits [35–37]. The natural resistance of the producer strain, *S. lydicus*, to Stl is due to the substitutions F485L and E486D (*E. coli* 545, 546) in the Stl pocket of the β subunit [19]. While the binding sites for Stl and Rif do not overlap, the Stl^R mutations display cross-resistance to Rif [34] and the Rif^R mutations result in cross-resistance to Stl, which arise likely due to allosteric effects [36, 37]. In addition, the Stl^R mutations in *rpo*B confer cross-resistance to the antibiotic microcin J25 [38] (see subsequent text).

Two antibiotics, tirandalydigin and tirandamycin, are structurally similar to Stl (Figure 12.3) and belong to the same acyl-tetramic acid family. Tirandalydigin was isolated from *Streptomyces tirandis* subsp. *umidus AB1006-A9* [39] and tirandamycin from *Streptomyces tirandis* sp. *Nov*. [40]. Both antibiotics inhibit the growth of the *S. aureus* ansamycin-resistant strain but not of the Stl^R strain [40]. The properties of tirandamycin are analogous to those of Stl. Tirandamycin inhibits bacterial RNAP but is 50-fold less potent than Stl (IC_{50} ~ 0.8 mM)) and is not active against eukaryotic RNAPs. The mechanism of action of these molecules is likely the same as Stl.

12.3.2
Lasso Peptides: Microcin j25 and Capistruin

Microcin J25 (MccJ25, MW ~ 2091 Da) is a cyclic 21-residue lasso peptide of (**Gly^1**-Gly-Ala-Gly-His-Val-Pro-**Glu^8**-Tyr-Phe^{10}-Val-Gly-Ile-Gly-Thr-Pro-Ile-Ser-Phe-Tyr^{20}-Gly) produced by *E. coli* strains harboring the pTUC plasmid encoding the four-gene cluster *mcjABCD* and was first isolated from the *E. coli* AY25 strain in 1992 [41]. The NMR structures of MccJ25 were resolved, showing an unusual ''lasso'' conformation with the covalent linkage between the α-amino group of Gly^1 and the γ-carboxyl of Glu^8 to form the ''lariat'' ring. The ''tail'' formed by residues 9–21 is sterically trapped in the ring [42, 43]. MccJ25 is active against

some gram-negative bacteria including the pathogenic *E. coli* O157:H7 strain [44], *Salmonella* and *Shigella* strains (MIC of 0.01–0.2 μg ml^{-1} [45, 46]). However, other gram-negative pathogen strains (e.g., *Pseudomonas aeruginosa* and *Moraxella catarrhalis*) are resistant.

MccJ25 inhibits both transcription initiation and elongation by *E. coli* RNAP at an IC_{50} value of 1–3 μM. The detailed mechanism of MccJ25 action is not well understood. The "cork in the bottle" model was proposed on the basis of biochemical and genetic data [47]. According to the model, MccJ25 binds within the secondary channel and obstructs it from NTP uptake [47]. However, MccJ25 inhibits the pyrophosphorolysis reaction performed by the RNAP catalytic site independently of NTP uptake, suggesting that the mechanism may not be a simple competition with incoming NTPs [48].

Two types of spontaneous MccJ25-resistance mutations have been identified. In the first class, the mutations are located in the *fhuA*, *exb*, *tonB*, and *sbmA* genes, which encode cell envelope proteins. The product of the *fhuA* gene, FhuA, is a principal receptor of MccJ25 and is responsible for its import [49]. In the second, the mutations are localized in the RNAP β′ subunit TL (*E. coli* T931I) [50]. Saturation mutagenesis allowed localization of ∼50 residues in the β and β′ subunits implicated in MccJ25 resistance [47]. Most of the $MccJ25^R$ mutations delineating the putative binding site are located in the secondary channel. No mutations leading to cross-resistance between Stl and MccJ25 were found in the β′ subunit. However, Stl^R mutations in the β subunit "Stl pocket" (Δ540–544) confers the resistance to MccJ25 [38], suggesting either overlap in the binding sites or similar mechanisms of action. In agreement with the idea of overlapping binding sites, binding of Stl and MccJ25 to RNAP was found to be mutually exclusive [31]. However, the effect of the mutations was proposed to be indirect because MccJ25 is supposed to bind in the secondary channel [38].

Recently, a new lasso peptide Capistruin (**G**^{1}TPGFQTP**D**^{9}ARVISRFGFN) was identified from *Burkholderia thailandensis* E264 [51]. Capistruin inhibits gram-negative bacteria (*E. coli*, *Burkholderia caledonica*, and *P. aeruginosa* with MIC values of 12–50 μM). This peptide is structurally similar to MccJ25 and was shown to inhibit wild-type *E. coli* RNAP but not MccJ25-resistant RNAP, suggesting similar binding sites and mechanisms of action [52].

12.3.3
CBR703 Series

A novel class of transcription inhibitors, the CBR703 series (*N*-hydroxy-*N*′-phenyl-3-trifluoromethyl-benzamidine, MW ∼ 280 Da), was isolated during high-throughput screening (HTS) of a large library of chemical compounds using *E. coli* RNAP core and holoenzyme transcription assays [53]. The CBR703 molecule specifically inhibited RNAP during transcription initiation and elongation with an IC_{50} of ∼10–20 μM, and no significant activity was observed against other nucleic acid polymerases from bacteria, viruses, or mammals. The inhibitor displayed low or no activity against gram-positive or gram-negative species (MIC of ∼128 μM) [53].

This low activity was proposed to be due to the specific efflux of the compound rather than to a problem in uptake or permeability as *E. coli* with *tolC* mutations was sensitive to CBR703 with an MIC of 16–24 μM. Modification of the progenitor compound yielded more effective derivatives, CBR9379 (IC_{50} of ~0.3 μM and MIC of 0.5 μM against *E. coli tolC* strain) and CBR9393 (IC_{50} of 2.5 μM and MIC of 8–16 μM for *E. coli tolC* strain). The most potent inhibitors exhibited activity even against the wild-type *E. coli* strains with an MIC of ~16–32 μM. CRB703 was shown to have bactericidal activity against bacterial persisters on *S. epidermis* biofilms [11]. These results indicate that these molecules are promising candidates for medical research and good scaffold for drug design.

Several CBR703-resistant and CBR703-dependent mutants were obtained by treatment of *E. coli tolC* cells with chemical mutagens. The mutations conferring resistance were clustered at the RNAP surface-exposed groove at the junction between the β subunit (P552L, P560L, G562V, R637C, R637S, S642R, and S642F) and the β′ subunit bridge-helix (P750L, F773V, and I774S). On the basis of biochemical and genetic data, it was proposed that CBR703 binds to the surface of RNAP and blocks all catalytic reactions of the active site, including NTP addition and PPi-driven hydrolysis. CBR703 does not affect the translocation of RNAP along the template. Because the CBR703 binding site is located away from the RNAP catalytic site, the mechanism of inhibition is noncompetitive and allosteric. CBR703 likely blocks BH conformational cycling required for NTP addition to the RNA chain; thus, its mechanism of action is reminiscent of that of Stl or Tgtx.

12.4 Antibiotics Blocking Promoter Complex Formation

12.4.1 Myxopyronin

Myxopyronin (Myx, MW ~ 417 Da, A form) is an α-pyrone antibiotic that was isolated from the myxobacterium *Myxococcus fulvus* Mxf50 in 1983 as a mixture of A and B forms [54]. Myx is active against a broad spectrum of gram-positive and gram-negative bacteria including *M. tuberculosis*, *S. aureus*, *Enterococcus faecium*, *P. aeruginosa*, and *E. coli* DH21 *tolC* with MIC values of $<12.5\,\mu g\,ml^{-1}$ [55]. Myx inhibits RNAP at an IC_{50} ~ 1 μM and does not inhibit eukaryotic RNAP II [54]. Complete chemical synthesis of Myx was described, and several analogs of desmethyl myxopyronin B with enhanced potency were produced [56]. Myx inhibits RNAP only during transcription initiation and has no effect on elongation. The antibiotic inhibits the formation of the RNAP open promoter complex when bound to RNAP before DNA but does not affect transcription if added to the preformed promoter complexes [55, 57]. The crystal structures of *T. thermophilus* RNAP in complex with Myx A [55] and its synthetic analog 7-desmethyl-Myx B [57] were resolved to resolution of 3 and 2.7 Å, respectively. Both structures showed that Myx binds within the main channel in the hydrophobic pocket formed by the β subunit

and the β′ subunit switch-1 and switch-2 regions. The binding site includes the β subunit residues 1271–1279 and 1322–1326 and the β′ subunit residues 334–345 (switch-2), 801–805, and 1323–1352 (switch-1).

Inactivation of the β′ switch-2 element plays a central role in Myx inhibition [58]. Two models explaining the mechanism of Myx action were proposed. The ''hinge jamming'' model [55] suggests that Myx prevents RNAP clamp opening, which in turn prevents loading of the downstream part of promoter DNA (promoter positions −11 to +15) into the main channel. The second model suggests that Myx hinders the β′ switch-2 refolding and prevents loading of the template single-strand DNA into the RNAP active site cleft [57]. Myx is considered a promising drug candidate because it has good activity against various pathogens and does not display cross-resistance with Rif owing to the distant binding sites. Some cross-resistance with other antibiotics targeting switch-2, corallopyronin, and ripostatin has been observed [55, 57].

12.4.2 Corallopyronin

Corallopyronin (Cor, MW ∼ 542 Da) is an α-pyrone antibiotic that was isolated as a mixture of three forms A, B, and C from the myxobacterium *Corallococcus coralloides* [59]. The chemical structure of Cor is similar to that of Myx and differs by a 7-carbon side chain extension of the deinone. Cor is active mainly against gram-positive bacteria with an MIC value of 0.1–10 μg ml^{-1} and at a much higher concentration against gram-negative bacteria (MIC value of >100 μg ml^{-1}) [59]. Accordingly, Cor inhibits RNAP at an IC_{50} value of ∼10 μM but does not inhibit eukaryotic RNAP [55, 59]. Biochemical studies showed that Cor acts essentially similarly to Myx and inhibits the formation of the open promoter complex [55]. Mutations conferring resistance to Cor revealed almost complete overlap with the binding site of Myx, encompassing the RNAP β′ switch-1 and switch-2 regions [58]. The only difference with Myx is the Cor^R mutation of the β subunit residue 1326, which may interact with the 7-carbon extension of Cor [58]. Cor does not exhibit cross-resistance with Rif [60], whereas strong cross-resistance with Myx and ripostatin was observed [55].

Recently, the Cor biosynthetic gene cluster (trans-AT-type mixed polyketide synthase (PKS)/*n*on-*r*ibosomal *p*eptide *s*ynthase (NRPS)) was characterized and the complete biosynthesis pathway of Cor A was deciphered [61]. Cor is not toxic in a mouse model. However, owing to the low activity and high frequency of spontaneous mutations observed in *S. aureus*, it is not considered a promising drug candidate [62].

12.4.3 Ripostatin

Ripostatins A and B (Rip, MW ∼ 496 Da) were isolated as a mixture from the culture supernatant of the *S. cellulosum* strain So ce377 [63]. Rip is a macrocyclic lactone carbonic acid containing an unsubstituted phenyl ring on a side chain and

is structurally different from Myx and Cor [63]. The chemical synthesis of Rip B was recently described [64]. Rip is a less potent antibiotic than Myx, active mostly on gram-positive bacteria: *S. aureus* (MIC of ~1 μg ml^{-1} [63]), *E. faecium*, and *C. difficile* [58]. Only one gram-negative bacteria, *Moraxella catarrhlis*, was reported to be sensitive to Rip [58]. The lack of activity on gram-negative species is due to the low permeability of their outer membrane as Rip displayed good activity on the *E. coli* D21f2TolC (*rfa tolC*) strain carrying the mutations of the genes involved in the membrane lipopolysaccharide (LPS) assembly [58]. Rip inhibits *in vitro* transcription by *E. coli* RNAP at an IC_{50} of ~1.5 μM but does not inhibit eukaryotic RNAP II [55, 63]. Considering the mutagenesis data showed overlap between the Myx and Rip binding sites, it was suggested that Rip inhibits transcription essentially in the same way as Myx, by blocking the formation of the open promoter complex [55]. Rip exhibits substantial cross-resistance with Myx and Cor but not with Rif [55].

12.4.4 Lipiarmycin

Lpm (MW ~ 1058 Da; also known as *fidaxomicin*, *tiacumicin B*, *OPT-80*, or *PAR-101*) is a macrocyclic glycoside antibiotic that was first isolated from *Actinoplanes deccanensis* [65, 66] as a mixture of A and B forms, and later identified in other actinomycetes: *Catellatospora* sp. Bp3323-81 [67], *Dactylosporangium aurantiacum* subsp. *hamdenensis* NRRL 18085, and *Micromonospora echinospora* subsp. *Armeniaca* [68]. Lpm is synthesized by a large *tia*-gene cluster encompassing ~31 genes [68]. Lpm is a narrow-spectrum antibiotic with antibacterial activity against gram-positive bacteria: *C. difficile* (MIC of <62 ng ml^{-1}) [69], *Bacillus subtilis*, and *Enterococcus faecalis* (MIC of 8–16 μg ml^{-1}) [70] and good activity against Rif^R forms of *M. tuberculosis* (MIC of <0.1 μg ml^{-1}) [67]. In addition, Lpm exhibits weak activity against gram-negative bacteria with an MIC of >11 μg ml^{-1} [58]. The frequency of spontaneous Lpm^R mutations in *S. aureus* was 3×10^{-8}, which is similar to that of Rif [58]. Lpm was approved for clinical use in 2011 for treatment of *C. difficile* infections under the name of Dificid® (Optimer Pharmaceuticals, USA).

Lpm blocks transcription initiation by RNAP with an IC_{50} of ~3–10 μM and is not active against eukaryotic RNAP II [71–73]. Biochemical studies showed that Lpm blocks isomerization from the closed to open promoter complex and does not affect transcription if added after the formation of the open promoter complex. The target site for Lpm on the RNAP surface was identified by the locations of the spontaneous Lpm^R mutations, which clustered at the N-terminal region of the β′ subunit (*E. coli* R99, P251, R337) and the C-terminal region of the β subunit (*E. coli* Q1256) [67, 72, 73]. Lpm binds within the upstream boundary of the main channel at the entry of the RNA-exit channel and targets several functional elements of the RNAP mobile clamp domain: the β′ subunit Zn-finger, Lid, switch-2, and β subunit switch-3 [73]. The binding site for Lpm partially overlaps the Myx binding site as both include the β′ switch-2 element. However, no significant cross-resistance between Lpm and Myx was observed [58]. It was proposed that Lpm may function

by blocking the switch-2 element dependent fitting of the template DNA into the active site [73]. However, the mechanisms of inhibition for Myx and Lpm are different because Lpm acts before Myx in the pathway of open complex formation [73, 74].

A specific feature of Lpm inhibition is its dependence on the σ subunit, thus resembling the properties of Rif. Indeed, Lpm was twofold more active against the RNAP holoenzyme than the core enzyme [73], suggesting that σ subunit may contribute to the formation of the binding site. Deletion of residues 513–519 in the σ subunit region 3.2 changes the sensitivity of RNAP to Lpm [73]. A similar effect was observed when σ was substituted by the SPO1 bacteriophage σ-like proteins gp28 and gp34, which lack the region 3.2 [75].

12.5
Inhibitors Hindering σ–Core Interactions

12.5.1
SB2 and Analogs (Phenyl-Furanyl-Rodanines)

A novel group of small molecular inhibitors was recently isolated from the ChemBridge™ chemical library using HTS based on the *in vitro* assay for σ–core interactions [76, 77]. SB2 (3-{5-[allyl-4-oxo-2-thioxo-1,3-thiazolidin-5-ylidene)methyl]-2-furyl}benzoic acid, MW ∼ 371 Da) and a structurally similar group of phenyl-furanyl-rodanine compounds (SB11, SB15, SB7, and SB8) was shown to inhibit holoenzyme assembly [76]. The compounds were active against gram-negative (*Pasteurella multocida*) (MIC of 1–4 μg ml^{-1}) and gram-positive bacteria, with the highest activity against gram-positive *Bacillus anthracis*, *Bacillus cereus*, *Streptococcus pneumoniae*, *S. aureus*, and *Staphylococcus epidermidis* (MIC of 0.3–2 μg ml^{-1}). SB2 was not active against *M. tuberculosis*, *Pseudomonas aeroginosa*, or wild-type *E. coli* strains (MIC of >33 μg ml^{-1}), but was active against *E. coli* D22, which bears a mutation in the *lpxC* gene that increases membrane permeability (MIC of 2 μg ml^{-1}). No cytotoxic effects on eukaryotic cells were observed. The phenyl–furanyl–rodanines compounds were active against *S. epidermidis* biofilms [78].

The SB2 mechanism of action is not yet understood. The molecule inhibits transcription by the RNAP holoenzyme (IC_{50} of ∼1 μM), likely by obstructing interactions between the σ subunit and catalytic core [77]. However, SB2 also inhibits σ-independent transcription performed by core RNAP on a poly(dA-dT) template (IC_{50} of <5 μM). Therefore, the target site of SB2 is located on the RNAP core. The mechanism of inhibition is likely allosteric because SB2 inhibits RNAP even after holoenzyme formation. No resistance mutations are known yet, preventing mapping of the inhibitor target site.

Another set of compounds targeting σ–core interactions (A5, A13, A8, and A17), some of which are structurally close to SB2, was isolated from the ChemBridge™ library during HTS based on the protein–protein luminescence resonance energy

transfer (LRET) assay [79]. The molecules inhibited *in vitro* transcription by the *E. coli* holoenzyme with an IC_{50} of ~25–100 μM more efficiently than the core enzyme and were not active against eukaryotic RNAPII. The molecules also inhibited σ–core interactions with an IC_{50} of ~3–28 μM. Two compounds, A5 and A13, were active against the wild-type *E. coli* strain and exhibited their highest level of activity against a permeable *E. coli* mutant [79].

A compound structurally similar to A5, fluoro-phenyl-styrene-sulfonamide (FPSS), was isolated recently during the HTS using a cell-based transcription assay [80]. FPSS specifically inhibits *in vivo* transcription (IC_{50} of ~3.5 μM) driven by the σ^B subunit (involved in stress response control and virulence) of *Listeria monocytogenes* and *B. subtilis*. However, the mechanism of action and the target site of this compound are not known yet.

12.6 Inhibitors with Unknown Mechanisms and Binding Sites

12.6.1 GE23077

GE23077 is a novel microbial metabolite isolated from the fermentation broth of an *Actinomadura* sp. and displays high inhibition activity against RNAP [81, 82]. GE23077 is a cyclic heptapeptide (MW ~ 803 Da), which was isolated as a mixture of four forms: A1, A2, B1, and B2. The GE23077-A2 and GE23077-B2 forms displayed approximately fivefold higher activity compared to that of A1 and B1 [82]. GE23077 inhibited transcription by *E. coli* and *B. subtilis* RNAPs with an IC_{50} value of ~25 nM. No activity against DNA pol and eukaryotic RNAP II was observed. Despite its high *in vitro* activity, GE23077 displayed poor antibacterial activity against gram-positive or gram-negative strains (MIC of $>200\,\mu g\,ml^{-1}$), with the exception of the gram-negative *M. catarrhalis* (MIC of $4–8\,\mu g\,ml^{-1}$), a significant human respiratory tract pathogen from the class of γ-proteobacteria. The inhibitor activity was higher against plasmolyzed *E. coli* cells (IC_{50} of ~2 μM), suggesting that the low membrane permeability is a cause for the lack of activity against bacterial cells [81].

GE23077 inhibits both σ-dependent and σ-independent transcription but is more active against the RNAP holoenzyme than the core enzyme (IC_{50} ~ 20 vs 100 nM, respectively) [81]. GE23077 inhibits a transcription initiation step following promoter binding but does not affect elongation. Thus, the GE23077 mechanism of inhibition may be similar to that of Rif. However, the inhibitor was active against RNAP bearing Rif^R substitutions, suggesting that there is no overlap in the binding sites [81]. Considering the high activity of GE23077 against RNAP, it can be considered as a good natural template for chemical modifications to extend its antibacterial spectrum to other pathogens. To date, chemical derivatization of the molecule has not resulted in improvement of the activity against bacteria other than *Moraxella* [83].

12.6.2
Ureidothiophene

Ureidothiophene (2-ureidothiophene-3-carboxylate, MW ~ 372.5 Da) is a low-molecular-weight inhibitor isolated in HTS of a commercial library of compounds using an *in vitro* transcription assay with the *S. aureus* RNAP holoenzyme [84]. The molecule displayed a high-level activity against RNAP with an IC_{50} of 1 μM, and exhibited a narrow spectrum of antibacterial activity against the *S. aureus* ATCC 13709 strain and *S. epidermis* with an MIC of 1–0.25 μg ml^{-1} but was not active against other strains of *Staphylococcus*. Ureidothiophene was not active against gram-negative bacteria. A series of its derivatives were obtained by substitution at position 3 (Figure 12.3), which allowed for improved specific activity with an IC_{50} between 0.04 and 1 μM. The compound was active against Rif^R strains of *Staphylococcus*, suggesting that the binding site and mechanism of inhibition differ from that of Rif.

12.7
Conclusions and Perspectives

12.7.1
Bacterial RNA Polymerase Inhibitors are a Valid Source of Clinical Drugs

The RNAP is targeted by a large number of natural compounds. Paradoxically, all of the binding sites for the known natural antibiotics are clustered in close proximity to the RNAP active center (Figure 12.2). However, we expect that other regulatory sites on the RNAP surface, for example, binding sites for transcriptional regulators, can be considered as possible targets for drug development. Indeed, the molecules found by HTS of chemical libraries seem to target sites distinct from those bound by the natural molecules. For example, no natural compound is known to affect σ–core interactions that are vital for the bacteria, but several of such molecules have been identified by HTS. Considering the richness of the natural compounds from bacterial sources, we expect that future screening for natural or synthetic molecules will lead to the finding of novel transcriptional inhibitors with new target sites. Furthermore, the current collection of inhibitors has not yet been fully explored and will likely provide scaffolds for designing new drugs.

12.7.2
The σ Subunit of RNAP Modulates Antibiotics Activity

Most of the transcription assays and screenings were performed with the holoenzyme containing the major housekeeping σ subunit. However, the number and structure of σs vary between bacterial species. The RNAP σ factors are basically grouped into two families: the *E. coli* σ^{70}-like housekeeping σs and the alternative σs (reviewed in [85]). Housekeeping σs are responsible for transcription of the essential genes during exponential growth, while alternative σ factors activate the

transcription of the specialized genes implicated in the stress response, virulence, and the switch to stationary growth phase or to a persistent state. The fact that variations in the structure of the σ subunit can modulate the sensitivity RNAP to the clinical drugs Rif and Lpm [15, 73], together with the finding of the FPSS compound that specifically targets the RNAP containing a particular σ factor [80], provides a proof of concept for the design of σ-specific drugs targeting specific transcription pathways (e.g., virulence genes). The divergence in RNAP core structures between bacterial species may also be responsible for the different sensitivities to antibiotics [86]. For example, *Thermus* RNAP is resistant to Lpm and ~100-fold less sensitive to Rif than *E. coli* RNAPs [14, 73]. Thus, a ''pathogen-specific'' approach should be applied when developing clinical drugs or screening for a new antimicrobial molecules. The antibiotics with high specificity to the particular forms of RNAPs (either containing specific σ factors or targeting only RNAP from pathogens) can be considered as future ''ecodrugs,'' which will not affect nonpathogenic bacteria essential for the biosphere but act specifically against the targeted pathogen.

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13
Inhibitors Targeting Riboswitches and Ribozymes

Isabella Moll, Attilio Fabbretti, Letizia Brandi, and Claudio O. Gualerzi

13.1
Introduction

Evidence accumulated over the past few decades has overshadowed the central role traditionally attributed to proteins in biological function and placed instead the RNA at the core of all fundamental biological processes. For instance, small RNA domains, such as riboswitches and ribozymes, have been shown to play important, if not essential, roles in a variety of cell functions, including regulation of gene expression. Currently, a large number of clinically relevant antibiotics target the ribosome, in particular its functional RNA component [1–4]; this indicates that, owing to its structural characteristics and complexity, the RNA is a suitable and efficient target for functional inhibition by small molecules. Similar to the interaction of proteins with small molecular ligands, the complex 3D structure of the RNA offers binding pockets and surfaces that provide hydrogen bonding, base stacking, ion pairing, and hydrophobic interactions for the specific binding of diverse compounds. In light of these premises, it is tempting to venture into novel paths in antibiotic research by exploiting small, functional RNA elements such as riboswitches and ribozymes as alternative targets for the design of novel antimicrobial compounds.

13.2
Riboswitches as Antibacterial Drug Targets

Riboswitches are noncoding, regulatory, metabolite-sensing RNA elements generally located within the 5-untranslated regions (UTRs) of certain transcripts. The intrinsic regulatory capacity of these small RNA elements was first identified at the beginning of the millennium during studies on the feedback regulation of vitamin biosynthesis operons in *Bacillus subtilis* [5, 6]. Subsequent studies have demonstrated the presence of riboswitches in a large number of bacteria, including many human pathogens, where they are involved in the regulation of genes essential for survival or virulence [7, 8].

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Up to now, over a dozen riboswitch classes have been identified, the majority of them controlling the expression of genes involved in transport and metabolism of small metabolite molecules that also represent their specific ligands. A typical riboswitch contains two distinct domains, a conserved aptamer domain and a less conserved expression platform [5–13].

Depending on the interaction of its aptamer with the cognate ligand, the riboswitch interconverts between two alternative, mutually exclusive structures, the "ligand-free" and the "ligand-bound" conformations, thereby controlling gene expression in two possible ways. In fact, while in some cases (the majority), the expression of the downstream genes is repressed on binding of the ligand to the aptamer, in other cases, they are activated when the riboswitch is ligand bound. Two examples of the latter type of mechanism are schematically illustrated in Figure 13.1. In the presence of its specific ligand, the RNA element harboring

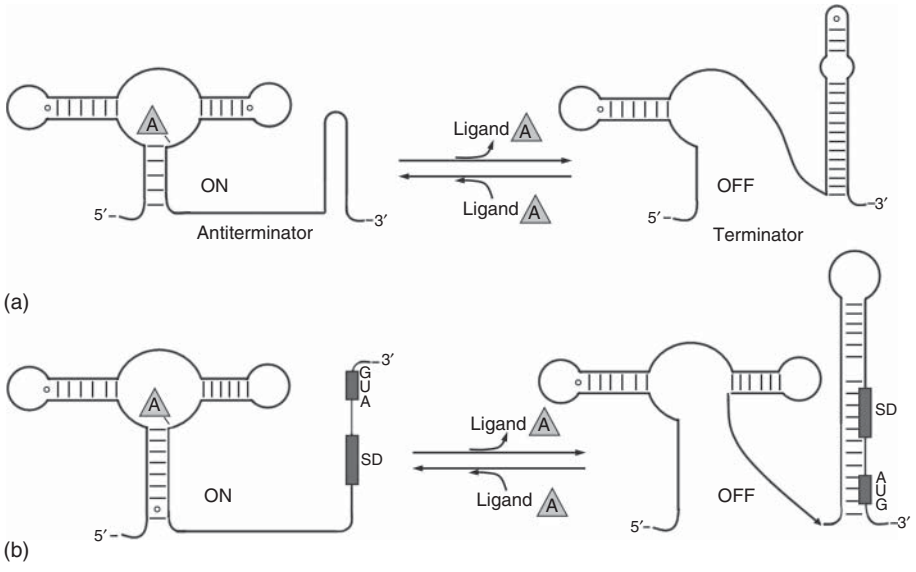

Figure 13.1 Riboswitch-mediated control of gene expression. Examples of mechanisms regulating gene expression operated by reversible interactions of purine riboswitches with their ligands. (a) Regulation of transcription due to a structural transition between ligand-bound (left) and ligand-free (right) riboswitch. The "ON" structure contains a transcription antiterminator that allows gene expression through the transcription of the downstream region. The "OFF" structure contains a transcription terminator stem that prevents RNA polymerase from transcribing the downstream genes. (b) Regulation of translation initiation due to a structural transition between the ligand-bound (left) and ligand-free (right) riboswitch. In the "ON" structure, the Shine–Dalgarno sequence and the initiation triplet (gray boxes) of the mRNA are in an open conformation, suitable for a productive interaction with the ribosomes, whereas in the "OFF" conformation, these elements become unavailable for the ribosomes. The two models of the figure reflect the situations found in the cases of (a) the gram-positive *Bacillus subtilis ydhL* Adenine [18] and (b) the gram-negative *Vibrio vulnificus add* Adenine riboswitch [19].

the riboswitch folds into an ''ON-state'' structure that does not interfere with the expression of the downstream genes placed under its control, whereas on dissociation of the metabolite ligand, the aptamer and the expression platform regions of the riboswitch undergo a structural rearrangement resulting in an ''OFF-state,'' which in turn leads to repression of gene expression. Although, in some rare cases, the regulation by riboswitches may involve an effect on splicing [14] or degradation of the transcript [15, 16], in most of the cases, the control of gene expression by riboswitches occurs either at the transcriptional or at the translational level [9–11]. However, at least in the case of the *Escherichia coli* lysC riboswitch, binding of lysine modulates translation initiation as well as the initial mRNA decay by affecting the RNA exposure to RNase E cleavage [17].

Transcriptional control is frequent in gram-positive bacteria and is operated through the induction by the ligand of either transcription termination (negative control) or, as in the example of Figure 13.1a, of antitermination (positive control). In gram-negative bacteria, riboswitches may influence gene expression by inhibiting or activating translation initiation through a mechanism that entails sequestering or exposing the translation initiation region (TIR) of the mRNA, thereby influencing ribosome binding (Figure 13.1b). In either transcriptional or translational control, the expression level of the downstream gene(s) is directly coupled in *cis* to the concentration of a specific compound that monitors the metabolic state of the cell, without the need for *trans*-acting protein factors or tRNAs [8–11].

Despite their high degree of conservation and the limited chemical diversity provided by the nucleotides, which constitute the building blocks of the RNA, the aptamer domains are folded in complex 3D structures, giving rise to binding pockets capable of binding with extremely high specificity molecules as small as a Mg^{2+} cation [13, 15] or glycine [20]. On the other hand, several riboswitch classes have evolved to recognize and bind the same *S*-adenosylmehionine (SAM) ligand. Although most of the riboswitches contain a single aptamer that binds a single ligand molecule, mRNAs containing tandem riboswitches have been found [21]. Indeed, the 5′UTR of *Bacillus clausii metE* mRNA contains one riboswitch responding to SAM and another responding to cobalamine (coenzyme B_{12}) so that its expression can be independently repressed by either ligand [21].

Another peculiar situation is found in *B. subtilis* where the glycine riboswitch, which controls the *gcvT* operon implicated in glycine degradation, contains two similar aptamers, separated by a linker, each binding one glycine molecule [20]. The physiological significance of the double glycine aptamer and their possible binding cooperativity [20, 22] remain uncertain [23].

Owing to the complex 3D structures of their aptamers, which contain specific binding pockets, intricate ligand–receptor interfaces are formed. Thus, almost all features of the ligands are recognized and extraordinary binding specificities are attained with discrimination levels of at least two–three orders of magnitude. Riboswitches are therefore able to respond to a large variety of small molecular ligands such as amino acids or coenzymes with extreme selectivity [18, 19, 24–28]. For instance, the pyrimidine-sensing helix of *thiM* is exquisitely tuned to select thiamine-pyrophosphate (TPP) by recognizing the H-bond donor and acceptors

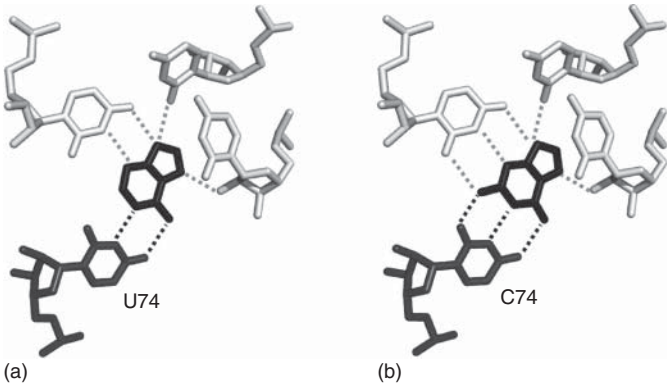

Figure 13.2 Ligand discrimination by purine riboswitches. Hydrogen bonds ensuring binding and binding specificity of (a) adenine and (b) guanine ligands by the corresponding riboswitches. The binding selectivity for adenine and guanine is ensured by Watson–Crick base pairings with U74 and C74, respectively. Further details are found in the text.

around its aminopyrimidine ring and by establishing TT-stacking interactions that may be sensitive to the electronic distribution of the rings; on the other hand, the presence of the pyrophosphate greatly increases the binding affinity of the riboswitch for its cognate ligand [24, 26, 28]. The discrimination between very closely related compounds, such as the purines guanine, adenine (Figure 13.2a,b), or 2-deoxyguanosine [18, 19, 25], is made possible by the establishment of specific Watson–Crick H-bonds so that gene expression can be regulated by the cognate ligand even in the presence of chemically and structurally similar molecules.

From the mechanistic aspect, the way in which riboswitches function could be based on either kinetic or thermodynamic (or both) properties of their interactions with the ligands [29–31]. However, in at least a subset of riboswitches, the actual control of gene expression seems to depend primarily on kinetic parameters such as the relative rate constants of RNA folding, ligand binding, and whatever biological event (e.g., transcription termination) is the object of the regulatory response. On the other hand, in practice, the thermodynamic stability of the complexes between riboswitches and natural ligands or their analogs is more important for the screening of ligand candidates and for the elucidation of the structures of the complexes. In fact, it is not uncommon that little or no relationship exists between the equilibrium thermodynamics of the complexes and their biological performance. Furthermore, it should be stressed that in many cases the K_d′s of the complexes are far too low to be of any use in tuning gene expression by a ligand whose cellular concentration is much higher (see subsequent text).

As mentioned earlier, the majority of the riboswitches are *cis*-acting elements that control downstream genes. However, examples of *trans*-acting riboswitches such as SAM, SreA, and SreB riboswitches of *Listeria monocytogenes* have also been described [32]. Although metabolite-binding RNAs, which may control important biochemical processes, have been found also in eukaryotic cells such as fungi and plants [14, 33, 34], riboswitches are found almost exclusively in prokaryotes. In light

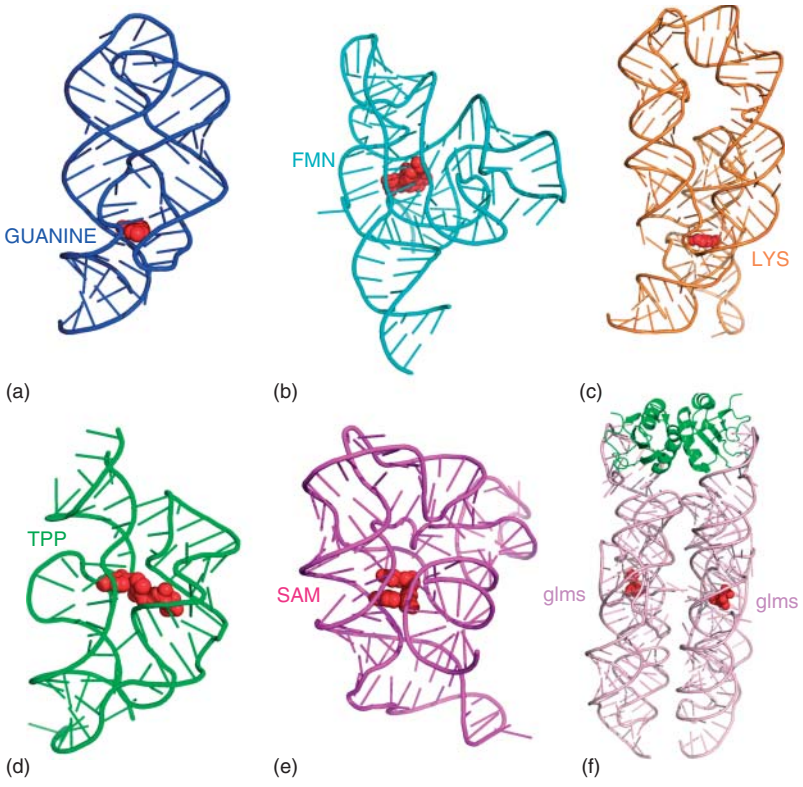

Figure 13.3 Three-dimensional structures of riboswitch–ligand and ribozyme–ligand complexes. (a) Guanine-bound G-riboswitch of *B. subtilis xpt* gene (pdb file 1Y27) [19]; (b) roseoflavin-bound FMN riboswitch of *Fusobacterium nucleatum impX* gene (pdb file 3F4H) [38]; (c) lysine-bound lysine-riboswitch of *Thermotoga maritima asd* gene (pdb file 3DIL); (d) thiamine pyrophosphate-bound TPP-riboswitch of *E. coli thiM* gene (pdb file 2HOL) [35]; (e) SAM-bound SAM-riboswitch of *B. subtilis yitJ* gene (pdb file 3NPB) [39]; and (f) GLC6P-bound *glmS*-ribozyme of *B. anthracis* (pdb file 3L3C) [40].

of this fact and of their aforementioned importance and properties, and of the fact that high-resolution 3D structures are available for a large number of them (e.g., Figure 13.3a–e), the riboswitches represent promising targets for the development of novel antibacterial drugs [10, 19, 35–39].

Indeed, riboswitch-binding molecules capable of inhibiting bacterial growth have been identified. Some of these molecules (e.g., roseoflavin, pyrithiamine, L-aminoethylcysteine, and D,L-oxalysine) (Figure 13.4) were existing inhibitors whose mechanisms remained unknown until their targets were identified as being riboswitches. In other cases, novel antimicrobial compounds capable of selectively targeting a riboswitch aptamer were obtained by rational design. This was made possible through screening and characterization of a limited number of ligand analogs (e.g., L-lysine analogs) synthesized exploiting the knowledge of the riboswitch 3D structures. Finally, in other cases, the riboswitch binders were identified [41, 42]

by the use of innovative fragment-based screening methods [41, 43–45]. In one case, 1300 fragments were screened and four molecules targeting *E. coli thi*M TPP riboswitch with fairly high affinity (22–60 μM) were identified (Figure 13.4 structures 1–4). However, in spite of their good binding to the aptamer, and possibly due to their inability to stabilize the P1–P1′ stem, none of the fragments proved effective

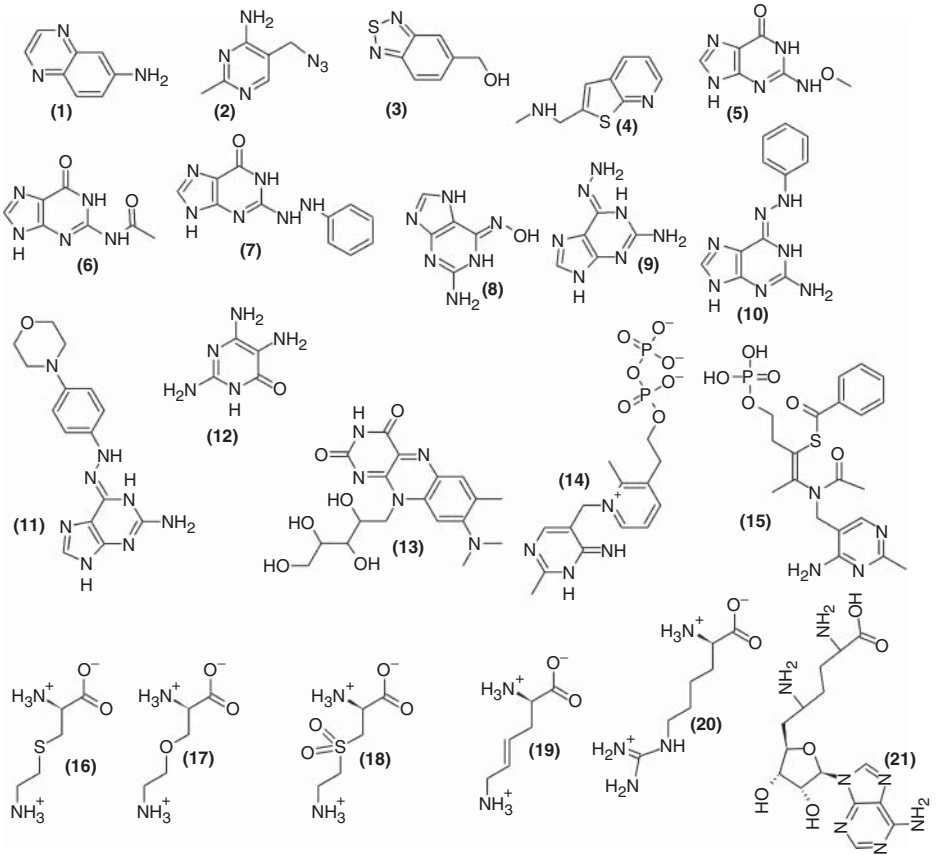

Figure 13.4 Chemical structures of some riboswitch ligands. The structures displayed in the figure are those of (**1**) quinoxalin-6-amine; (**2**) 5-(azidomethyl)-2-methylpyrimidin-4-amine; (**3**) 2,1,3-benzothiadiazol-5-ylmethanol; (**4**) *N*-methyl-1-(thieno[2,3-*b*]pyridin-2-yl)methanamine; (**5**) 2-(methoxyamino)-1,9-dihydro-6*H*-purin-6-one (G2); (**6**) *N*-(6-oxo-6,9-dihydro-1*H*-purin-2-yl)acetamide (G4); (**7**) 2-(2-phenylhydrazinyl)-1,9-dihydro-6*H*-purin-6-one (G6); (**8**) (6*Z*)-6-(hydroxyimino)-6,7-dihydro-1*H*-purin-2-amine (G7); (**9**) (6*Z*)-6-hydrazinylidene-6,9-dihydro-1*H*-purin-2-amine (G11); (**10**) (6*Z*)-6-(2-phenylhydrazinylidene)-6,9-dihydro-1*H*-purin-2-amine (G14); (**11**) (6*Z*)-6-{2-[4-(morpholin-4-yl)phenyl]hydrazinylidene}-6,9-dihydro-1*H*-purin-2-amine (G16); (**12**) PC1; (**13**) roseoflavin (8-dimethyl-amino-8-demethyl-D-riboflavin); (**14**) pyrithiamine pyrophosphate (PTPP); (**15**) benfotiamine (or *S*-benzoylthiamine); (**16**) S-(2-aminoethyl)-L-cysteine (AEC); (**17**) L-4-oxalysine; (**18**) L-3-[(2-aminoethyl)-sulfonyl]-alanine; (**19**) DL-*trans*-2, 6-diamino-4-hexenoic acid; (**20**) L-homoarginine; and (**21**) sinefungin (SFG).

in inhibiting the expression platform in an *in vitro* transcription–translation system [46]. Thus, the identified fragments can only be regarded as useful starting points for improvement in the design of new inhibitors and for future studies on the relationships between structure and activity of this riboswitch.

13.2.1 Purine Riboswitches

The purine-responsive riboswitches represent an important group of RNA structures involved in the regulation of a large number of genes (>2% of the total in *B. subtilis*) mainly implicated in purine metabolism and transport (reviewed in [25]). The purines are recognized by four classes of riboswitches, three of which have rather similar primary and secondary structures, whereas another class of riboswitch, endowed with a different structure, binds 7-aminomethyl-7-deazaguanine (preQ1). As detailed earlier (Figure 13.2a,b), these riboswitches recognize with high selectivity their cognate ligands such as guanine, adenine, 2′-deoxyguanosine, xanthine, and hypoxanthine that are bound with K_d′s in the nanomolar range [18, 19, 25, 47].

Guanine riboswitches represent one example of several tandem riboswitches, whose existence was recently reported [21]. Indeed, in approximately 300 cases, the guanine aptamer resides in tandem with *ykkC* (Figure 13.5), an RNA element predicted by bioinformatic analyses to be an aptamer [48, 49] but whose ligand has not yet been identified [50]. In the absence of guanine, an antiterminator

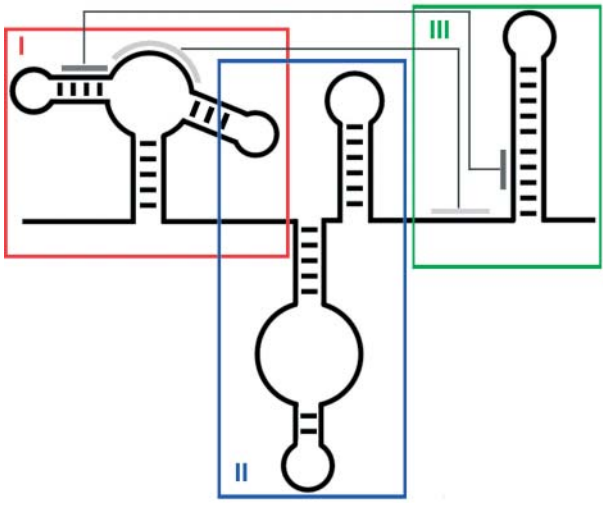

Figure 13.5 Schematic representation of the tandem arrangement of a guanine riboswitch with a ykkC element. The model that reflects the structure of the *purE* mRNA of *Moorella thermoacetica* [25] indicates the guanine-binding aptamer (red box I), the transcription termination stem-loop (green box II) and the predicted aptamer *yccK* (blue box III). Further details can be found in the text.

structure is formed, whereas binding of guanine to the aptamer determines the formation of a transcription terminator stem. On the other hand, binding of a ligand (whatever its identity might turn out to be) to the *ykkC* motif is predicted to prevent the formation of the terminator stem, in competition with the guanine-bound riboswitch structure. In the context of this book on antibiotics, the *ykkC* aptamer may be of particular interest insofar as it is believed to switch on, among others, also those genes involved in multidrug-resistance efflux pumps and detoxification systems. Should this prediction prove correct, the potential advantages derived from interfering with this riboswitch would be obvious.

In light of their importance, distribution in nature, and of the knowledge so far acquired on their overall properties, including their atomic structures, purine riboswitches are considered suitable targets for new anti-infectives. In fact, the crystal structures of the *add* A-riboswitch and *xpt* G-riboswitch aptamer modules (Figure 13.3b), which distinguish between adenine and guanine with exquisite specificity and modulate expression of two different sets of genes, have been elucidated [19]. These riboswitches are endowed with forklike architectures, with the prongs kept in a parallel orientation by interactions established by the hairpin loops. The purines are bound in a pocket inside an internal bubble and held in place by hydrogen bonds with conserved nucleotides, whereas the specific recognition of adenine versus guanine is ensured by Watson–Crick base pairings (Figure 13.2a,b). This structural information, as well as other atomic structures of riboswitches, is being exploited for the rational design of riboswitch-binding molecules (see subsequent text).

Accordingly, several guanine analogs have been prepared by rational design, taking into account the fact that the atomic-resolution structure of a *B. subtilis* guanine riboswitch aptamer indicates that there is free space adjacent to C2 and C6 of the ligand. Thus, a total of 16 modified guanine molecules bearing different substituents at these two positions were synthesized; the structures of some of them are shown (Figure 13.4 structures 5–11) [51].

A number of guanine analogs constructed in this way display a fairly high affinity for the aptamer. Compared to the K_d of guanine (~5 nM), G7, G11 had only slightly higher K_ds (20 and 8 nM, respectively), whereas G4 displayed a 10-fold higher affinity for the aptamer. However, when the effect of these various analogs on bacterial growth was tested, it became clear that little or no relationship exists between the microbiological activity of the various molecules and the K_ds of their complexes with the aptamer. In fact, neither G4 nor G11, which has higher affinity for the riboswitch, display any relevant effect on bacterial growth, whereas G2, G6, G14, and G15, which have strongly reduced affinity for the RNA target (>600-fold in the case of G6) were found to inhibit bacterial growth efficiently. The results obtained by the use of *lacZ* reporter gene placed under the control of the riboswitch allowed the riddle to be solved by showing that only G7 (Figure 13.4 structure 8) is capable of targeting the riboswitch *in vivo*, whereas the other molecules owed their microbiological activity to the inhibition of other cell functions. This premise was confirmed, at least in the case of G6; in fact, the analysis of mutations giving rise to

resistance to this molecule showed sequence modifications within the terminator stem of the expression platform of the *pbuE* adenine riboswitch [51].

Other studies demonstrated that not only purine analogs but also modified pyrimidines bearing amino groups at positions 5 and 6 and that may mimic the N7 and N9 of purines can bind to the purine riboswitch with fairly high affinity [52]. That some of these pyrimidine derivatives offer good opportunities to become antibiotic candidates is indicated by the compound named PC1 (Figure 13.4, structure 12). Indeed, this molecule was shown to interact with guanine riboswitches and to display bactericidal activity against a variety of bacterial strains, including several clinical strains exhibiting multiple drug resistance, when the target riboswitch controls the expression of *guaA*, a gene coding for a GMP synthetase. Furthermore, in a mouse model, the administration of PC1 was found to reduce a mammary gland infection by *Staphylococcus aureus* [53].

13.2.2
c-di-GMP (Bis-3′-5′-Cyclic Dimeric Guanosine Monophosphate) Riboswitch

Cyclic diguanosyl-5′-monophosphate (c-di-GMP) is an important signaling molecule in bacterial cells, just like cyclic AMP, ppGpp, and likely cyclic-di-AMP. This ''second messenger'' is implicated in a large number of cell functions, some of which, as in biofilm formation and virulence expression, are of particular relevance in the context of bacterial pathogenicity. In addition to protein receptors of c-di-GMP, RNA motifs that respond to c-di-GMP have also been detected among the 22 bacterial-candidate-structured RNAs identified by comparative genomic analyses [49]. These elements are found in association with genes involved in c-di-GMP production, degradation, and signaling. At least two distinct riboswitch classes (I and II) respond to c-di-GMP (Figure 13.6a,b) [54, 55]. Several hundred class I c-di-GMP-binding riboswitches and approximately 50 class II riboswitches have been identified in diverse bacterial species. While both classes are present in a few bacteria, several species of Clostridia have exclusively class II c-di-GMP riboswitches. The dangerous pathogen *Clostridium difficile*, which is one of these bacteria, contains four riboswitches of class II, one them being a regulator of a group I intron [55]. Both types of riboswitches have an exceptionally high affinity for their ligand, with K_ds in the picomolar range.

The 3D structure of the riboswitch present upstream of the *Vibrio cholerae tfoX* gene has been solved in its complex with c-di-GMP [58]. Overall, the structure of this riboswitch resembles that of the purine riboswitches [19, 47], with the ligand-binding pocket being also located at a three-helix junction; but the molecular nature of the binding pockets, which accommodate either the purines or the c-di-GMP, is completely different. Two separate binding pockets are implicated in the recognition of the two nucleobases of c-di-GMP, and both of them are required for high-affinity binding.

The twofold symmetric ligand is asymmetrically bound at a three-helix junction of the monomeric RNA by both canonical and noncanonical base pairing and intercalation accounts for the discrimination of this RNA aptamer against another

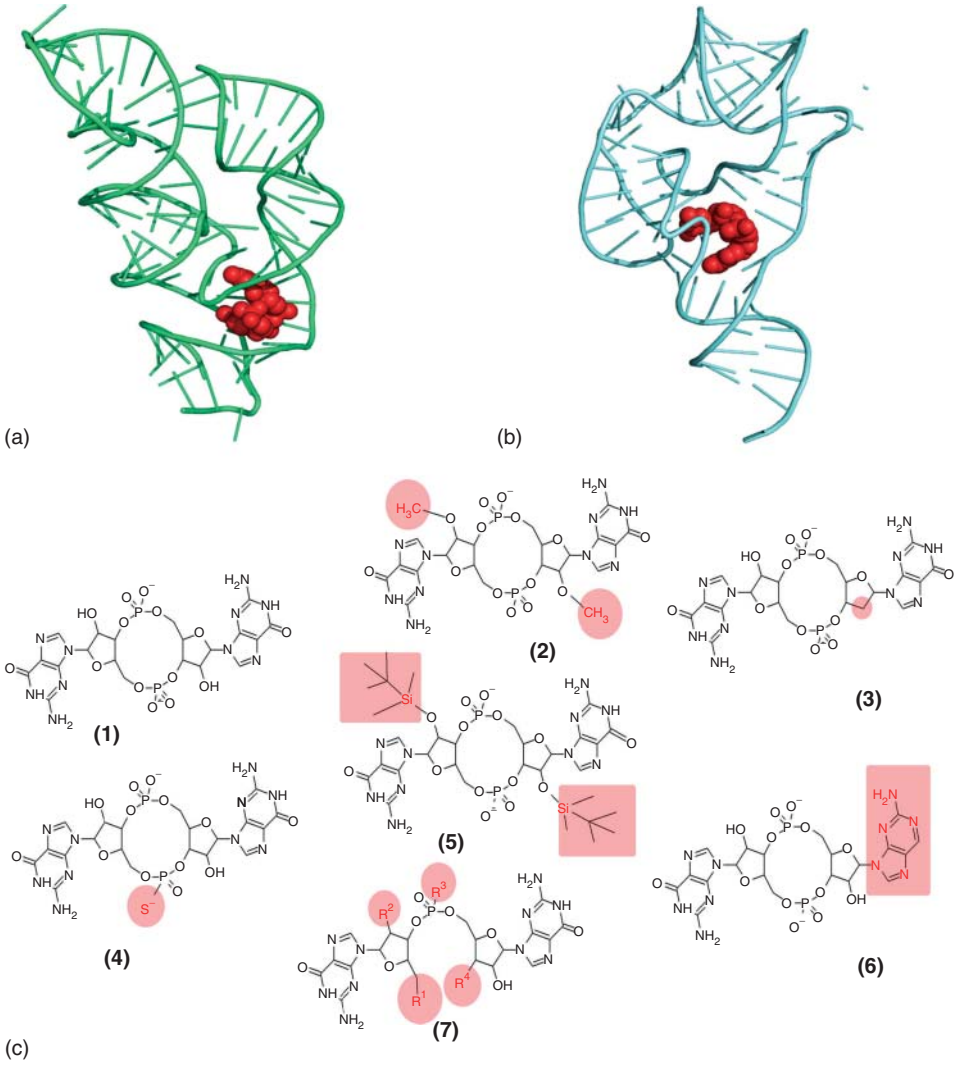

Figure 13.6 Three-dimensional structures of class I and class II c-di-GMP riboswitch and chemical structures of some of their ligands. Crystal structures of riboswitch aptamer domains of (a) a representative of class I riboswitch (green), namely, the Vc2 aptamer from *Vibrio cholera* bound to c-di-GMP (red) (PDB ID 3MXH) [56] and (b) a representative of class II riboswitch (cyan), namely, the Cac-1-2 aptamer from *Clostridium acetobutylicum* bound to c-di-GMP (red) (PDB ID 3Q3Z) [57]; (c) chemical structures of c-di-GMP (structure **1**) and its derivatives 2′-O-methyl c-di-GMP (structure **2**), c-dGpGp (structure **3**), c-GpGps (structure **4**), 2′-OTBDMS CDG (structure **5**), c-GpAp (structure **6**), and a linear c-di-GMP analog (structure **7**). The positions and in some cases the chemical structures of the substituents are highlighted (pink).

similar second messenger such as cyclic-diadenylate (c-di-AMP) [59]. Biophysical and biochemical data indicate that a large-scale structural rearrangement of the core three-helix junction occurs on c-di-GMP binding [58]. Thus, c-di-GMP riboswitch belongs to the group of riboswitches (e.g., *B. subtilis metE* SAM-I; *Fusobacterium nucleatum/B. subtilis queC* preQ1; and *E. coli thiM/Arabidopsis thaliana thiC* TPP), which undergo extensive conformational changes on ligand binding, unlike those riboswitches (e.g., *B. subtilis/F. nucleatum* FMN; *B. subtilis xpt* guanine; *Thermoanaerobacter tencongensis* preQ1; and *Thermotoga maritima asd* lysine) in which only minor structural alterations occur [60].

In another study, the structures of class I and class II aptamers and their interactions with the c-di-GMP ligand were elucidated at the atomic level [57, 61]. The riboswitch of class I has a y-shaped structure consisting of three helices, unlike class II ribozymes that display a compact structure containing a kink-turn and a pseudoknot. Also, in these structures, the symmetrical ligand is bound asymmetrically in both types of aptamers. In both cases, c-di-GMP participates in the RNA structures; in the aptamer of class I, one guanine is paired in a duplex, whereas in class II, it is part of a triplex helix formed with a pseudoknot. Furthermore, unlike the case of the class II aptamer, the class I aptamer establishes contacts with both guanines and with the phosphodiester backbone. Overall, these interactions, together with the stacking contacts established with three adenosine nucleotides of the aptamer, can account for the very low (pmolar) K_ds of the RNA–ligand complexes and for the higher affinity of c-di-GMP for class I aptamer compared to class II riboswitch [57]. Guanine recognition is ensured by Watson–Crick and Hoogsteen pairs and by noncanonical pairings in class I and class II aptamers, respectively [57, 61].

The c-di-GMP riboswitches represent promising targets for new inhibitors in light of their wide distribution in the bacterial kingdom and of their involvement in the control of virulence expression of many pathogens.

Indeed, exploiting the acquired structural information that the ribose 2′ hydroxyl groups of the c-di-GMP are involved in H-bondings with class I, a 2′-O-methyl analog of c-di-GMP (Figure 13.6c structure 2) was designed with the prediction that it would not bind to this aptamer but only to the class II aptamer. When empirically tested in binding competition assays, the analog was found to bind to class II but not to class I riboswitch, thereby confirming the expectation [57].

In another study, a series of both circular (Figure 13.6 structures 3–6) and linear (Figure 13.6 structure 7) c-di-GMP analogs were synthesized and tested for their capacity to bind to class I and class II riboswitches and to affect transcription termination [62]. When tested with class II aptamer, all circular analogs synthesized (Figure 13.6c structures 2–6) displayed a strong affinity for the RNA, whereas only the analogs having minor modifications (Figure 13.6c structures 3 and 4) bound to the class I aptamer with high affinity, unlike one with bulkier substituents (Figure 13.6c structure 5) or one in which a guanine had been substituted by an adenine (Figure 13.6c structure 6). A somewhat similar situation was observed with the linear c-di-GMP analogs. However, when tested for their effect on transcription termination, none of the analogs, not even those which efficiently bound to the

aptamers, displayed an appreciable activity in affecting transcription. These results were explained by the inability of the analogs to compete kinetically with the termination or antitermination events [62].

Overall, the results obtained with the c-di-GMP analogs [57, 62–64] confirm the premise derived from the crystallographic studies, namely, that binding to class I riboswitch has much more stringent structural requirements compared to class II.

13.2.3 FMN Riboswitches

The riboflavin biosynthetic pathway is considered a valid target for broad-spectrum antibiotics [65]. The FMN riboswitches are fairly high conserved RNA elements (RFN elements) frequently found in the 5′UTR of bacterial mRNAs encoding proteins involved in the biosynthesis and transport of FMN and are therefore potential targets for new inhibitors [5, 6, 66, 67]. In *B. subtilis*, this riboswitch causes premature transcription termination [5, 6] within the 5′UTR of the *ribDEAHT* and affects translation by precluding ribosome access to the TIR of *ypaA* mRNA [6, 67]. The 3D structure of the complexes of *Fusobacterium nucleatum* – FMN riboswitch with FMN, riboflavin, and its analog roseoflavin – have been elucidated by X-ray crystallography [38]. In the complexes, the ligands are asymmetrically bound within a pocket located at a six-stem junction of the aptamer, which forms a butterfly-shaped scaffold (Figure 13.3b). The binding specificity is ensured by the interactions of the heteronuclear tricyclic ring of the isoalloxazine, which is part of the flavine, and by contacts with the RNA phosphate, both direct and mediated by Mg^{2+} cations [38]. FMN-like ligands with anti-infective properties have been designed on the basis of the structure of the FMN-binding pocket [38].

As mentioned, roseoflavin (8-dimethyl-amino-8-demethyl-D-riboflavin) (Figure 13.4 structure 13), an already known antibacterial and antifungal antibiotic [68] whose mechanism of action had not been elucidated, turned out to target the FMN riboswitch. This molecule is synthesized by *Streptomyces davawensis* and is an analog of riboflavin (vitamin B_2) [69]. The *ribB* FMN riboswitch of the roseoflavin producer *S. davawensis*, in contrast to that of the closely related bacterium *Streptomyces coelicolor*, is able to discriminate between two very similar ligands [FMN and roseoflavin mononucleotide (RoFMN)], yielding different responses. As a result, *S. davawensis* is roseoflavin resistant, unlike *S. coelicolor* which is roseoflavin sensitive [70].

In the cytoplasm of the target cell, roseoflavin is converted to RoFMN and roseoflavin adenine dinucleotide, which bind to the FMN aptamer [38]; as a consequence of this interaction, roseoflavin inhibits both biosynthesis and transport of riboflavin, two processes that are under the control of the FMN riboswitch. Accordingly, roseoflavin was found to downregulate the expression of *ribD* riboswitch-*lacZ* reporter genes in *B. subtilis* [71] and very low concentrations of this antibiotic were found to inhibit growth of *L. monocytogenes*, a gram-positive pathogen that lacks the ability to synthesize riboflavin *de novo*. The fact that base substitutions in the FMN aptamer confer resistance to roseoflavin further indicates that the observed

inhibition is due to a direct binding of this molecule to the FMN riboswitch Rli96 of this organism [72]. While these results seem to induce optimism as to the potential of roseoflavin to function as an anti-riboswitch antibiotic, other data suggest a much more cautious attitude. In fact, through a mechanism independent of the FMN riboswitch, roseoflavin was found to enhance the expression of the virulence genes *hly*, *actA*, *plcA*, and *prfA*. Thus, unlike the canonical ligand riboflavin, which reduces the *L. monocytogenes* virulence, roseoflavin was found to increase both virulence and infection ability of the pathogen [72]. Despite this, the possibility of chemically synthesizing a large number of flavin analogs, possibly starting from roseoflavin as a lead structure, and the use of the efficient screening methods devised so far, offers many opportunities to select inhibitors with the desired properties and having the potential to be developed into novel anti-infectives [73].

Several gram-negative pathogens cannot transport riboflavin and fully depend on riboflavin biosynthesis, a process often regulated by FMN riboswitches. Thus, a ligand capable of repressing riboflavin biosynthesis by targeting this riboswitch should be lethal for these cells.

13.2.4
Thiamine Pyrophosphate (TPP) Riboswitch

TPP, a derivative of vitamin B_1, is selectively recognized by RNA elements known as *TPP riboswitches* [5, 26]. These are the most widespread class of riboswitches known because they are ubiquitous in the bacterial genomes and have been found also in eukaryotes such as *A. thaliana* and *Aspergillus oryzae*, where an intron in the 5′UTR of the thiamine biosynthesis gene *thiA* contains two regions forming part of a TPP riboswitch that controls the splicing of the transcript, an essential event for *thiA* gene expression and thiamine synthesis [74].

In the bacterial kingdom (*E. coli* and *B. subtilis*), TPP regulates its own production by binding to the 3′ UTR of *thiC* mRNA where the riboswitch is localized [5, 26]. The riboswitch forms specific and high-affinity (K_ds in the nanomolar range) complexes with its natural ligand (TPP or thiamine diphosphate) and also with natural or synthetic TPP analogs. Of particular importance among these analogs is pyrithiamine. This molecule targets TPP riboswitches and inhibits growth of several bacterial [26, 28, 75] and fungal [76] species. Similar to thiamine, pyrithiamine is readily phosphorylated inside the cells to yield pyrithiamine pyrophosphate (PTPP) [77, 78]. PTPP, which differs from TPP in that a pyridinium ring replaces the thiazole ring (Figure 13.4 structure 14), binds *in vitro* to several TPP riboswitches with an affinity comparable to that of TPP. Most important, PTPP represses *in vivo* the expression of a reporter gene fused to a TPP riboswitch, a finding which also suggests that, in its phosphorylated form, pyrithiamine inhibits bacterial or fungal growth by repressing one or more TPP-riboswitch-regulated genes. Furthermore, several pyrithiamine-resistant strains of *B. subtilis*, *E. coli*, and *A. oryzae* were shown to carry mutations in a conserved region of TPP riboswitches, which normally regulate the expression of thiamine biosynthetic genes. Moreover, *in vitro*

ligand binding to the riboswitch and repression of a reporter gene regulated by the riboswitch were prevented by the mutations.

Another thiamine analog capable of binding the TPP riboswitch is benfotiamine (or *S*-benzoylthiamine) [35]. This is a synthetic S-acyl derivative of thiamine (Figure 13.4 structure 15) licensed as a dietary supplement and prescribed for treating sciatica and other similar nerve affections; however, there are no indications as to its possible antibiotic activity.

To gain structural information that could guide the design of novel antimicrobials targeting TPP-sensing riboswitches, the crystal structure of the complexes between the *A. thaliana* TPP-riboswitch and PTPP and oxythiamine pyrophosphate (OTPP) has been solved at 2.9 Å resolution [34, 79]. The 3D structure of a ligand-bound TPP riboswitch (Figure 13.3d) indicates that the specific recognition of the ligand is ensured by conserved residues located within two highly distorted parallel ''sensor'' helices; The aminopyrimidine ring of TPP provides donor and acceptor groups for H-bonding with various components of the aptamer and engages in TT-stacking interactions that may be sensitive to the electronics of the ring. In the OTPP–riboswitch complex, the pyrimidine ring is stabilized in its enol form to retain key interactions with a guanosine of the riboswitch, which are observed also in the TPP complex. In the complex of the riboswitch with PTPP, another guanine undergoes a conformational change to cradle the pyridine ring of PTPP. The central thiazole ring of TPP is surrounded by several water molecules and should not be directly recognized by the receptor so as to make the identity of the central ring not essential for ligand binding. Although this is the likely reason for the aptamer binding of PTPP, which differs from TPP only in the nature of the central ring, more recent studies have shown that the central thiazolium ring of TPP could be more important for ligand recognition than previously thought. It could contribute electrostatic interactions and allow the *in vivo* discrimination between thiamine (and its phosphate esters) and other aminopyrimidines [24]. The pyrophosphate moiety of the ligands is not strictly necessary for modulation of gene expression but only to ensure submicromolar K_ds to the complex. Thus, the riboswitch aptamer is able to recognize a monophosphate ligand in a manner similar to how it recognizes the β-phosphate of TPP. When the ligand is the monophosphorylated compound, the RNA elements that recognize the thiamine and phosphate moieties of the ligand move closer together. In the complex with pyrithiamine, the binding site for the pyrophosphate is largely unstructured. Overall, the structural data explain the mechanism of resistance to pyrithiamine and suggest that in addition to TPP and PTPP the binding pocket could accommodate other PTPP-like ligands.

Thus, following structure-based rational design, a series of TPP analogs could be synthesized in the future and tested for their capacity to act as ligands of TPP riboswitches and possibly to inhibit riboswitch-dependent functions and bacterial growth. New-generation screening methods, such as the medium-throughput fragment-based methodology for screening libraries of small molecules developed to detect *E. coli thiM* riboswitch ligands, will prove very useful in this enterprise. Indeed, this method has already allowed the identification of several high-affinity ligands from which potential riboswitch inhibitors could be developed [41, 42].

13.2.5
Lysine Riboswitch

Lysine riboswitches are RNA elements whose aptamer is a highly selective and sensitive sensor of the lysine concentration. The 3D structures of a *T. maritima* lysine riboswitch has been determined in the apo-form as well as in complexes with lysine [37, 80]. In addition, the structures of the complexes of the riboswitch with lysine analogs, which had been shown to bind with fairly high affinity to the aptamer (see subsequent text), were determined. These ligands were S-(2-aminoethyl)-L-cysteine (AEC) (Figure 13.4 structure 16) and L-4-oxalysine (Figure 13.4 structure 17), two compounds in which the C4 position of the natural amino acid is replaced by sulfur and oxygen, respectively; the others were L-homoarginine and N6-1-iminoethyl-L-lysine, two analogs in which the ε-amine of lysine is replaced by a guanidinium group and its methyl-substituted variant, respectively (not shown). As described later, despite their somewhat reduced affinity for the riboswitch, all these analogs display antibacterial activity.

The atomic structure of the lysine-bound riboswitch is rather complex, containing a bundle of two colinearly stacked helices and three helices, two of which are also colinearly stacked. The helices are connected by a five-way junction. An elongated and rather rigid binding pocket, whose geometry is such as to precisely fit the lysine, is located at this junction. In its binding pocket, the ligand is ''sandwiched'' between RNA bases and establishes both direct and K^+-mediated hydrogen bonds with the minor groove edges of purine bases and with the 2′OH of the ribose [80]. An interesting feature of the structure is that one of its stems contains an unconventional kink-turn that is involved in a loop–loop interaction essential for lysine binding [81]. Comparison of the crystallographic structures of the lysine-bound and apo-form of the riboswitch reveals that the overall pattern of base interactions is the same, with only some minor perturbations. Thus, as described earlier, the lysine ribozyme belongs to the group of ribozymes that do not undergo major conformational rearrangements on ligand binding [60]. However, chemical probing and single-molecule fluorescence resonance energy transfer (FRET) experiments clearly indicate that to perform its function the lysine-binding aptamer undergoes structural opening/closing transitions on interaction with its ligand, thereby underlining the importance of RNA dynamics in the operational mechanisms of riboswitches [82]. The rate constants of these structural transitions governing the binding and dissociation of lysine to and from the *B. subtilis lysC* aptamer have been determined and the apparent dissociation constant for lysine ($K_d = 0.25$ mM) calculated from these data [83] turned out to be far higher than previously reported ($K_d = 1–3$ μM) at 10 mM Mg^{2+}. However, as it seems unlikely that a riboswitch might respond to a ligand such as lysine whose free concentration inside bacteria (between 0.1 and 10 mM) is orders of magnitude higher than its K_d, it appears clear that the kinetic more than the thermodynamic aspects of the riboswitch–ligand interaction are of fundamental importance for regulation of gene expression by these RNA elements.

Lysine riboswitches are found in the 5′ UTR of mRNAs and are devoted to the modulation of the expression of genes implicated in lysine metabolism as a function of the lysine concentration [84] Thus, lysine-mimicking molecules capable of binding to the riboswitch in place of the natural ligand are expected to reduce the expression of aspartokinase and cause lysine starvation together with other metabolic disorders.

Because lysine biosynthesis and transport could be suppressed by compounds binding to a lysine riboswitch, lysine analogs have been designed and synthesized with the hope of finding new inhibitors potentially useful in antibacterial therapy. Indeed, several lysine analogs that bind to riboswitches *in vitro* and interfere with bacterial growth have been found [85]. Twelve lysine analogs were tested for their capacity to bind the *B. subtilis lysC* riboswitch, to affect bacterial growth, and to repress the expression of a reporter gene placed under the control of the riboswitch [85]. Five of the molecules tested (i.e., L-3-[(2-aminoethyl)-sulfonyl]-alanine (Figure 13.4 structure 18); L-4-oxalysine (Figure 13.4 structure 17); DL-*trans*-2, 6-diamino-4-hexenoic acid (Figure 13.4 structure 19); L-homoarginine (Figure 13.4 structure 20); and *N*6-1-iminoethyl-L-lysine) were found to bind with K_ds ranging from 1 to 13 μM, values somewhat higher compared to that of lysine (360 nM). Overall, these results indicate that at least some modifications at position C4 and addition of some functional groups on N6 of lysine do not impair the binding to the aptamer.

When the biological activity of these five riboswitch-binding lysine analogs were tested, two of them, for unknown reasons, did not display any microbiological activity, unlike the other three (i.e., L-3-[(2-aminoethyl)-sulfonyl]-alanine; L-4-oxalysine and DL-*trans*-2, 6-diamino-4-hexenoic acid) which completely inhibited bacterial growth as well as the expression of the reporter gene (β-galactosidase). Overall, a good correlation was observed between K_ds, reporter gene repression, and MICs of these molecules [85].

This correlation as well as the fact that mutations within the RNA aptamer confer AEC- and DL-4-oxalysine-resistant phenotypes in both *B. subtilis* [86] and *E. coli* [87] could be interpreted to mean that the inhibition is due to binding to the riboswitch. However, the identification of the *in vivo* target of AEC and DL-4-oxalysine is not easy. In fact, the antibacterial effect of these analogs could well be an inhibition of the lysyl-tRNA synthetase (LysRS). Indeed, it has been shown that in *E. coli* the inability of LysRS to discriminate between AEC and lysine [88] is the main reason for the toxic effects of AEC. In fact, the misaminoacylation of $tRNA_{lys}$ results in the incorporation of AEC into proteins in the place of lysine. The AEC resistance caused by mutations in the riboswitch has a simple explanation. Loss of lysine-dependent regulation of the lysine biosynthetic enzymes determines an increase in the intracellular lysine concentration, and this circumstance allows lysine to effectively outcompete AEC for binding to LysRS [88]. Finally, it should be borne in mind that even if lysine biosynthesis is blocked by interference with *lysC* expression, some bacteria have riboswitch-independent backup pathways to produce lysine. In addition, even if these pathways also are completely repressed by riboswitch inhibitors the bacteria could nevertheless survive by importing lysine

from the environment, which normally contains sizable amounts of this amino acid. This occurrence has been verified in the case of *B. anthracis*, whose growth is sensitive to inhibitors targeting the lysine riboswitch in lysine-free media, but not in lysine-rich medium [85].

13.2.6 SAM (S-Adenosylmethionine) Riboswitches

Methionine (Met) and cysteine (Cys) biosynthetic operons are upregulated by Met starvation through leader sequences (S-box) and feedback regulated by SAM. The nascent S-box RNA binds SAM and induces the formation of a transcription terminator that blocks gene expression. Thus, the S-box leader RNA behaves like a typical SAM-sensitive riboswitch. Since the initial discovery of these RNA elements [89], many more SAM riboswitches have been detected so that at present at least seven classes of these RNA elements are known (i.e., SAM-I, SAM-II, SAM-III, SAM-IV, SAM-I/IV, SAM-V, and SAM/SAH). However, it seems likely that more riboswiches of this type will be discovered in the future [9, 89–96].

Some of these riboswitches, such as SAM-I, which is the one most frequently found in bacterial mRNAs, display very high selectivity for the ligand. In fact, they can discriminate between SAM, which is an important and abundant metabolite, and other similar molecules such as *S*-adenosylhomocysteine (SAH), the toxic breakdown product of SAM [27, 97]. However, not all classes of SAM riboswitches display this capacity to discriminate between similar ligands; for instance, the SAM/SAH riboswitch binds SAH with very high efficiency [96].

The 3D structure of the SAM I riboswitch of *Thermoanaerobacter tengcongensis* bound to its SAM ligand has been solved by X-ray crystallography at 2.9 Å resolution. The RNA was shown to possess a complex architecture, capable of recognizing, either directly or indirectly, almost all functional groups of SAM. Structural communication between aptamer and expression platform is ensured by ligand-induced tertiary interactions involving one of the RNA helices [98].

In another crystallographic study, the 3D structure of *Enterococcus faecalis* S (MK)-box riboswitch, which regulates gene expression at the translational level, was solved at 2.2 Å resolution [99]. In the structure of this riboswitch, SAM is bound through extensive interactions of its adenosine and sulfonium moieties at a three-way junction present in the Y-shaped RNA element. Remarkably, the mRNA SD sequence also participates directly in the recognition of the ligand. In contrast to SAM, the SAM analog SAH was shown to establish fewer contacts with the RNA.

The 3D structure of the complete SAM-II riboswitch containing both aptamer and expression platform has also been solved; in the same study, chemical probing experiments shed light on the structural changes occurring in response to SAM binding, which make the mRNA inaccessible to the ribosomes and result in translational downregulation [100].

More recently, a fairly high-resolution (2.4–2.9 Å) 3D structure of the SAM-I riboswitch was obtained [97], making use also of sequence variants of the RNA. The molecular basis for the binding specificity was shown to reside in the positively

charged sulfonium group of SAM, which establishes electrostatic interactions with two carbonyl oxygens [U7(O2) and U88(O2)] of two conserved A–U pairs in one of the RNA helices. In the same study, the interaction of the riboswitch with the SAM analogs SAH and sinefungin (SFG) (Figure 13.4 structure 21) was also investigated; the latter is an antimicrobial, antifungal agent produced by *Streptomyces griseolus* and is a competitive inhibitor of methyltransferases. Despite the fact that the adenosyl and amino acid main-chain moieties of SFG form the same set of hydrogen bonds observed in the complex with SAM, SFG binds about two orders of magnitude more weakly than SAM. This was attributed to the inability of the SFG ligand to adjust its position to optimize its electrostatic interactions with the aptamer [97].

13.3 Ribozymes as Antibacterial Drug Targets

In contrast to riboswitches, ribozymes are catalytically active RNA molecules, involved in various biological activities, including the regulation of gene expression [101–103]. Ribozymes perform a phosphoryl transfer reaction, catalyzing the cleavage and/or ligation of the RNA phosphodiester backbone. For self-cleaving ribozymes, such as the hepatitis delta virus (HDV) ribozyme, the hairpin, and the hammerhead ribozymes (HHRs), the general mechanism of the site-specific cleavage is similar to that of many protein RNases in which a 2′oxygen nucleophile attacks the adjacent phosphate in the RNA backbone, resulting in cleavage products with 2′,3′-cyclic phosphate and 5′ hydroxyl termini [104, 105]. Employing base-pairing and tertiary interactions, ribozyme cleavage occurs at very specific sequences within the catalytic core [104]. The smallest ribozyme (50–150 nucleotides) is the HHR found in all kingdoms of life [106, 107]. Although the majority of ribozymes are *cis*-acting, for some HHRs also a *trans*-acting cleavage activity was identified [103, 108]. In contrast to self-cleaving ribozymes, self-splicing ribozymes, such as the Group I and Group II introns, catalyze a phosphodiester-cleavage reaction that produces 5′-phosphate and 3′-hydroxyl termini followed by the respective ligation [105, 109, 110].

A particular group of ribozymes are the metabolite-responsive ribozymes. Similar to riboswitches, these functional RNA elements can bind and sense small metabolites that introduce allosteric changes that trigger RNA self-cleavage activity, which plays an important role in the regulation of gene expression [101]. One of the best-studied metabolite-responsive ribozymes, which is conserved in a variety of gram-positive bacteria, is located upstream of the *glmS* transcript of *B. subtilis* encoding the glucosamine-6-phosphate synthetase [101]. The *glmS* ribozyme, whose structure is shown in Figure 13.3f, catalyzes a site-specific cleavage in the *glmS* mRNA in the presence of glucosamine-6-phosphate (GlcN6P), a pivotal metabolite in bacterial cell-wall biosynthesis. The cleavage results in the formation of a 5′-OH terminus on the *glmS* mRNA that represents the target for RNase J1, a 5′-3′ exonuclease present in gram-positive bacteria [111]. Thus, *glmS* mRNA stability is regulated by a negative feedback in a ligand-responsive manner [112]. It is intriguing to note that, in contrast to riboswitches where ligand binding introduces

allosteric changes, several lines of evidence support the model that the metabolite GlcN6P serves as a cofactor essential for the catalytic activity of the *glmS* ribozyme (reviewed in [113]).

That ribozymes could be targeted by antibiotics was discovered almost 20 years ago, when a set of aminoglycosides that interact with the decoding region of *E. coli* 16S rRNA was found to inhibit the second step of splicing of the T4 phage-derived td intron as well as the splicing of other Group I introns, whereas Group II introns were not inhibited [114–118]. The similarity between rRNA and Group I introns with respect to their binding affinity and specificity for these antibiotics suggested that the recognition is based on evolutionarily conserved structures, and it was surmised that antibiotics and RNA may have coevolved in the formation of the modern ribosome [114]. Subsequently, it was discovered that aminoglycosides can also inhibit the activity of hammerheads [119, 120]. In fact, neomycin (Figure 13.7 structure 1) was found to reduce the rate of cleavage of two hammerheads with $K_i = 13.5\,\mu M$. The inhibition was found to be due to a preferential interaction of the antibiotic with the enzyme–substrate complex for which the ammonium ions of neomycin are important. The antibiotic–hammerhead interaction resulted in a stabilization of the ground state of the complex and in a destabilization of the transition state of the cleavage step [119].

Also, the hairpin ribozyme [121], the human HDV ribozyme [122, 123], and RNaseP, both in the presence and the absence of the RNaseP protein [124], were later found to be inhibited by aminoglycosides (neomycin B being the strongest inhibitor of RNaseP) as well as by peptide and tetracycline classes of antibiotics. In all cases, the reported K_i were in the micromolar range. In light of the fact that RNaseP is essential for cell survival, as explained in greater detail in Chapter 14, this ribozyme indeed represents a potentially ideal target for the development of new drugs, possibly starting from aminoglycosides as lead compounds.

As to the mechanism by which aminoglycosides inhibit ribozymes, a general model for ribozyme inhibition by cationic antibiotics was put forward [125] after the indication, by several follow-up studies, that inhibition of splicing by Group I introns depends on the amino groups of neomycin B and that aminoglycosides compete for binding with ions for functionally important divalent metal ions and may actually displace several essential Mg^{2+} ions from the catalytic core of the HHR [115, 116, 119].

The corollary of the aforementioned, serendipitous discoveries that micromolar concentrations of known antibiotics such as streptomycin, neomycin, viomycins (Figure 13.7 structure 2), tetracycline (Figure 13.7 structure 3), and so on, inhibit Group I introns as well as other ribozymes was the suggestion that catalytic RNA could serve as a target for inhibitors that could be used for the therapeutic treatment of infections caused by both bacterial and fungal pathogens whose life cycle is regulated by/depends on ribozymes [114, 126].

Accordingly, different approaches have been devised with the aim of designing and synthesizing new molecules specifically targeting regulatory and catalytic RNAs or of identifying natural products endowed with these properties. In one of these approaches, an *in vitro* splicing test of *Pneumocystis carinii* Group I intron was

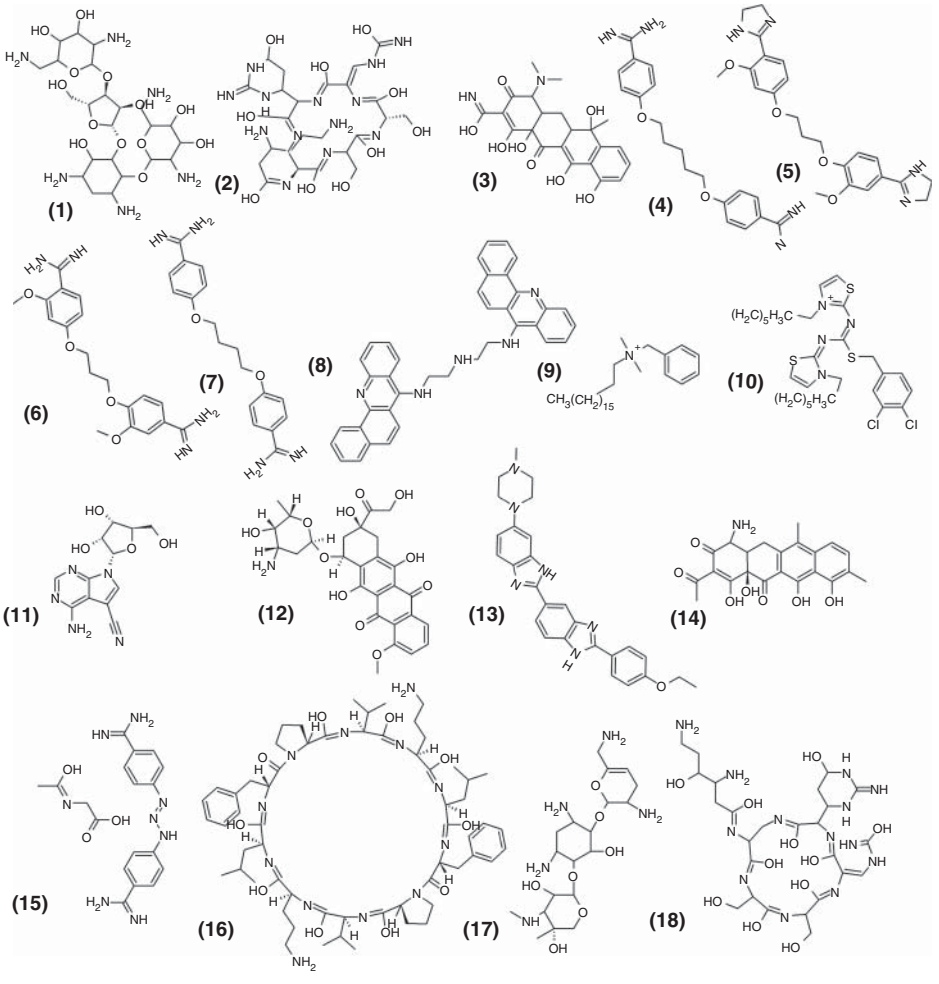

Figure 13.7 Chemical structures of some ribozyme ligands. The structures displayed in the figure are those of (**1**) neomycin; (**2**) viomycin (tuberactinomycin B); (**3**) tetracycline; (**4**) pentamidine; (**5**) DIMP; (**6**) DAMP; (**7**) butamidine; (**8**) N^1-(benzo[c]acridin-7-yl)-N^2-(2-(benzo[c]acridin-7-ylamino)ethyl)ethane-1,2-diamine; (**9**) *N*-benzyl-*N*,*N*-dimethyloctadecan-1-ammonium; (**10**) 2-((E)-((E)-1-(3,4-dichlorobenzylthio)-2-(3-heptylthiazol-2(3H)ylidene)ethylidene) amino)-3-heptylthiazol-3-ium; (**11**) toyocamycin; (**12**) adriamycin RDF (doxorubicin); (**13**) bisbenzimide; (**14**) chelocardin; (**15**) diminazene aceturate; (**16**) gramicidin S; (**17**) 5-epi-sisomicin; and (**18**) tuberactinomycin A.

used to detect inhibitors of this reaction. The results demonstrated that in addition to aminoglycosides, tetracycline, L-arginine, and ethidium bromide, pentamidine and a series of pentamidine analogs inhibited the first step of the splicing reaction [126, 127]. Pentamidine (Figure 13.7 structure 4), designated as an ''orphan drug'' by the FDA, is an aromatic diamidine used since the late 1930s in antiprotozoan

therapy, being the drug of choice against African trypanosomiasis and antimony-resistant leishmaniasis. In addition, this antibiotic is active against pathogenic yeasts such as *Candida albicans* and is used in the antifungal therapy of *P. carinii* pneumonia [128, 129]. Similar to other diamidines, despite being poorly membrane permeable, pentamidine is accumulated to high concentrations inside parasites such as *Leishmania* spp. and trypanosomes and is also concentrated several 100-fold inside *Plasmodium falciparum*-infected erythrocytes, displaying *in vitro* antimalarial activity [130–132]. As to its mechanism of inhibition, pentamidine affects a large number of cell activities (e.g., folic acid synthesis, trypsin-like proteases) and targets different types of macromolecules, including DNA to which it binds *via* an interaction with the minor groove [133, 134]. However, as the antimicrobial effect of pentamidine and its derivatives against *P. carinii* does not correlate well with their DNA-binding activity and with other molecular interactions tested [135], it seems likely that RNA functions are the effective cellular targets of these antibiotics and that ribozyme inhibition is just one aspect of this activity [136]. Indeed, it has been shown that pentamidine inhibits Group I intron splicing not only in *P. carinii* but also in yeasts such as *C. albicans* [136] and *S. cervisiae* [137] as a consequence of the mechanism by which it binds to the RNA, establishing large hydrophobic interactions by inserting its aromatic rings into the stacked base pairs of the RNA helices and thereby disrupting the secondary structure of the RNA [138]. The same mechanism is responsible for the binding of pentamidine to tRNAs and for the consequent inhibition of aminoacylation [139].

Unfortunately, the therapeutic use of pentamidine is limited by its demonstrated nephrotoxicity [140] so that potentially more effective and safer pentamidine derivatives such as 1,3-di(4-imidazolino-2-methoxyphenoxy)propane (DIMP), 1,3-bis(4-amidino-2-methoxyphenoxy)propane (DAMP), and butamidine (Figure 13.7 structures **5–7**) have been synthesized and tested [135, 141].

Other innovative, reproducible, rapid, and sensitive *in vitro* filtration high-throughput screening (HTS) assays based on *P. carinii* and bacteriophage T4 Group I introns were developed to detect molecules targeting and inhibiting self-splicing. These tests were applied to a library of approximately 150 000 small organic molecules at Parke-Davis Research Laboratory belonging at the time to Warner-Lambert Co. and a polyamine, an organic ammonium salt, and a thiazol (Figure 13.7 structures 8–10) were identified as three nonnucleic acid, nonaminoglycoside-based compounds able to inhibit the two-step intron splicing and RNA ligation [142, 143]. The determined IC_{50} (~10 μM) of these molecules was approximately 50-fold higher than that displayed by guanosine [143] but, to the best of our knowledge, no follow-up has since occurred.

Another HTS cell-based assay in which expression of a luciferase (luc) reporter is controlled by ribozyme sequences was developed to identify inhibitors of RNA self-cleavage in mammalian cells. On screening almost 60 000 small molecules, 15 inhibitors of ribozyme self-cleavage were identified, the most effective being the antifungal compound toyocamycin (Figure 13.7 structure **11**) an adenine analog produced by several *Streptomyces* spp. as well as some other nucleoside analogs

[144, 145]. As it turned out, toyocamycin inhibits RNA self-splicing also *in vivo* and could find an application as an antitumor agent [146].

In another study, a collection of 2000 actinomycetes extracts as well as a small library of 96 known RNA binding antibiotics were screened to identify inhibitors of HHR activity. This screening was carried out using an innovative fluorescence-based HTS assay by which several hundred different ribozyme reactions per day can be analyzed [147]. Approximately 100 positive samples were identified within the collection of the actinomyces extracts which had been preliminarily subjected to filtration through a membrane with a 3000 Da cutoff. On the other hand, 16 compounds belonging to the group of known antibiotics were found to inhibit the HHR-cleavage reaction. The antibiotics displaying the strongest inhibition ($K_i < 20\,\mu M$) were adriamycin RDF (doxorubicin), bisbenzimide, chelocardin, diminazene aceturate, gramicidin S, 5-epi-sisomicin (Figure 13.7 structures 12–17) and the two tuberactinomycins of the viomycin family, TubA (Figure 13.7 Structure 18) and TubB (Figure 13.7 Structure 2). The highest level of inhibition ($K_i = 110$ nM), approximately 350-fold more effective than neomycin B ($K_i = 38\,\mu M$) was detected with TubB. However, when these HHR inhibitors identified *in vitro* were tested *in vivo* for their activity inside cells using the U3 snoRNA-HHR hybrid "*cis*-snorbozyme" [148], with the exception of TubA, none of these molecules was found to be active in yeast cells. As to the reasons why compounds that are potent inhibitors *in vitro* do not show activity *in vivo*, the authors speculate that these results could be attributed either to the impermeability of the cell membrane or simply to the inaccessibility of the ribozyme *in vivo*. However, at least in the cases of TubB and bisbenzimide, the authors argue that due to the high toxicity of these molecules (100 and 10 μM, respectively), it is likely that the intracellular concentration of these compounds necessary for inhibition cannot be reached. On the other hand, at least TubA displayed a modest yet reproducible and concentration-dependent inhibition.

As mentioned above, also the metabolite-responsive *glmS* ribozyme was suggested to represent a promising target for antibacterial drug development, insofar as it regulates the synthesis of GlcN6P, an essential substrate for cell wall formation [149, 150]. Furthermore, as the *glmS* ribozyme is highly conserved throughout gram-positive bacteria, drugs that target this ribozyme could be effective against a broad range of pathogenic bacteria. Thus, high-throughput methods to screen small compound libraries for molecules that activate the *glmS* ribozyme were recently described [149, 151].

Unfortunately, in contrast to the expectations, these screenings did not reveal any novel compound interfering with the ribozyme activity, except for glucosamine, an analog of the natural ligand GlcN6P.

13.4 Concluding Remarks and Future Perspectives

Two, apparently unrelated circumstances, namely, the growing evidence for the central role played by RNA in all vital processes and the realization that innovative strategies must be employed to develop new anti-infectives may converge to

determine a challenging scenario in the quest of new antibiotics which are absolutely necessary to cope with the emergence of multidrug-resistant, killer super-bugs [152].

The functional and structural characterization of RNA elements such as riboswitches and ribozymes, if not in its infancy, is still in a stage of rapid growth. Thus, it seems legitimate to expect that in the next few years more exhaustive information about the genetic networks under riboswitch control and the molecular aspects of RNA–ligand interactions will become available. As outlined above, riboswitches and ribozymes are constituted by simple RNA elements assembled to make rapid, sophisticated, energetically cost-efficient genetic decisions, or to perform catalytic functions without involving protein factors, a circumstance which should make the rational design of ligand molecules a rather straightforward task, provided that the atomic structures of their complexes of the corresponding ligands are known.

Furthermore, the extensive sequence conservation displayed by the riboswitch aptamer domains renders the riboswitches readily identifiable by bioinformatic analyses so that the number of identified riboswitches is steadily rising. As new riboswitches are being discovered from genomic data analyses and sequence comparisons [48, 49, 91, 96], new potential riboswitch targets and new opportunities arise for the development of ligands capable of inhibiting the expression of bacterial functions which are crucial for pathogenicity. As an example, a widespread RNA motif named GEMM (genes for the environment, for membranes and for motility) having the characteristics of a riboswitch was detected in association with genes important for natural competence in *V. cholerae* and the use of metal ions as electron acceptors in *Geobacter sulfurreducens* [49]. The GEMM was subsequently shown to bind the ''second messenger'' c-di-GMP that modulates the expression of a large number of genes implicated in bacterial responses to various environmental cues [54]. Thus, a molecule that would bind to *V. cholerae* riboswitch (Vc1) in place of or in competition with its canonical ligand c-di-GMP would likely neutralize the virulence of this pathogen because this riboswitch controls *gbpA*, a gene implicated in virulence expression; it encodes a sugar-binding protein necessary for the colonization of mammalian intestines [153].

Bioinformatic investigations based on microbial genomics and metabolomics are expected to detect new genetic circuits subject to the regulatory control of RNA elements and will offer the essential background for the development of innovative HTS assays for the detection of natural or synthetic inhibitors while structural biology data will fuel the rational design of appropriate inhibitors. Along these lines, novel inspiring approaches like the one devised by Tran and Disney [154] may represent a breakthrough toward the construction of novel RNA-targeting inhibitors. To identify RNA structures representing privileged targets for small ligands, these authors have used a library versus library multidimensional combinatorial HTS and screened $\sim 3 \times 10^6$ combinations of RNA motif–small molecule interactions [154].

From the data reviewed in this chapter, it should become apparent that inhibitors targeting small regulatory RNA structures represent a promising tool in fighting

emerging multiresistant microbial pathogens and "superbugs." Nevertheless, finding good RNA ligands and inhibitors of select functions *in vitro* and *in vivo* does not exhaust the task. As with all other drugs, a number of pharmacological issues such as toxicity, bioavailability, pharmacokinetics, and so on, must be tackled and solved. Furthermore, as in the case of all other antibiotics, the possible emergence of resistance represents a possible drawback.

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14
Targeting Ribonuclease P

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14.1
Introduction

Ribonuclease P (RNase P) is the enzyme responsible for the maturation of the 5′ end of the precursor tRNAs (pre-tRNAs) [1]. Recently, it has been reported that human nuclear RNase P contributes also to the efficient transcription of various small noncoding RNA genes transcribed by RNA polymerase I and III, such as rRNAs, tRNAs, 5S rRNA, and U6 snRNA [2, 3]. Bacterial RNase P, in almost every case, is a ribonucleoprotein (RNP), consisting of a catalytic RNA (rapid plasma reagin, RPR and a protein subunit (P protein) [1]. On the other hand, archaeal and eukaryal RNase P enzymes contain an RNA subunit, slightly different from their bacterial counterpart and an increasing number of protein subunits as we step up on the evolutionary ladder.

Two exceptions that challenge the RNP character of RNase P have been reported: the human mitochondrial RNase P and RNase P from *Arabidopsis thaliana* chloroplasts, which does not contain any RNA and the enzymatic activity has been totally shifted to proteins [4, 5]. RNase P existence in three kingdoms of life, in comparison with its varying composition among different classes of organisms, give to this enzyme the perspective to be considered as a very effective drug target.

The RPR from bacteria, some achaea, the lower eukaryote *Giardia lamblia*, and humans are *bona fide* ribozymes, and they exhibit catalytic activity in the absence of the protein components *in vitro* [1, 6, 7]. Divalent metal ions and predominantly Mg^{2+} ions are absolutely required for RNase P function, assuming a dual role: they confer to electrostatic repulsion between the negatively charged phosphates of the polynucleotide backbone for proper folding and they participate in the reaction mechanism by activating the attacking nucleophile (H_2O) and stabilizing transition-state intermediates [8]. This important feature of RPR makes it the main target for several antibiotics. Moreover, the RPR as a polyelectrolyte and negatively charged molecule can create the essential environment to interact with any positively charged molecules or cations. Thus, any molecule that could affect or

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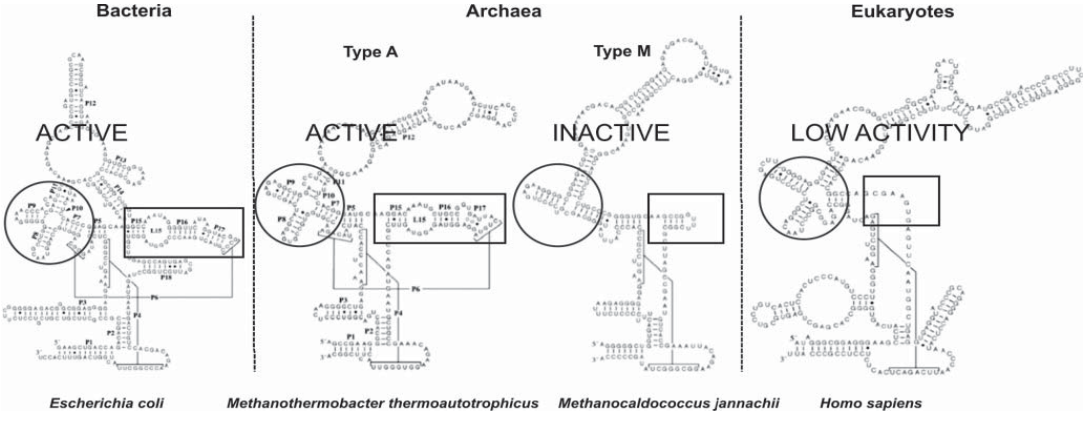

Figure 14.1 Secondary structures of RPRs from the three different domains of life. Bacterial and archaeal of type A RPRs exhibit RNase P activity (ribozymes), while the archaeal of type M are inactive. Among the eukaryal RPRs, only RPR from human and *Giardia lamblia* exhibit a weak catalytic activity. The basic structural differences are in the stem P15 (gray box) and P8 (gray circle).

interfere in the RPR interactions with the P protein subunits could be considered as a putative modulator of RNase P activity.

The crystal structure of bacterial RNase P complex with its universal substrate pre-tRNA [9] revealed the interactions among RPR, P protein subunit, and pre-tRNA, and provided the structural basis of putative mechanisms of action of RNase P modulators. The protein component seems to interact with the 5′ leader sequence of the pre-tRNA and help to position it correctly in the complex [10, 11]. Another important feature for the substrate recognition interaction is the base pairing between the 3′-complementary chromatic acclimation (CCA) end of the pre-tRNA and the complement sequence in the L15 loop in the RPR (Figure 14.1). However, it is worth noticing that this interaction is not conserved in organisms where CCA addition occurs posttranscriptionally. Moreover, in eukaryotes and some archaea the ribonucleotide reductase (RNR) lacks the L15 loop (Figure 14.1) [12], and the presence of more protein components may substitute its function in this intermolecular interaction.

RNase P has been extensively studied in the past two decades as a promising drug target using either antisense strategies or classical inhibitors (Table 14.1). The versatility of eukaryotic RNase P, together with its RNP idiosyncrasy, makes RNase P an ideal *in vitro* molecular model for the study of various important inhibitors. It has been characterized by many as a small ribosome, owing to its resemblance with the cell's translational machinery.

Throughout the past 20 years, many accumulated data suggest that RNase P can be affected and possibly regulated by many molecules of various sizes and properties, either *in vitro* or in the cellular environment. However, the exact mechanism of their action still remains elusive. Future structural studies, which will also include eukaryotic enzymes, may provide a rationale for the mechanism of action of many compounds, as well as the synthesis and development of novel drugs.

14.2
Targeting RNase P with Antisense Strategies

Since the first discovery of the catalytic properties of P RNA, many antisense strategies have been deployed as tools to target it. Initially, they were based mainly in modified oligonucleotides which fulfilled specific biochemical characteristics such as persistent resistance to hydrolysis by nucleases. Among the modified molecules that have been synthesized and used to inactivate or inhibit P RNA is LNA (locked nucleic acid, with a methylene bridge between the 2′-oxygen and 4′-carbon atom) and PNA (peptide nucleic acid, the deoxyribose phosphate backbone is replaced by polyamide linkages). DNA, RNA, LNA, and PNA variants of the 14-mer inhibitor against nucleotides 291–304 of *Escherichia coli* P RNA were used to study the antisense inhibitors behavior [13]. The strongest inhibitory effect was observed by RNA and LNA duplexes, followed by PNA and DNA. Interestingly, the PNA 14-mer could also inhibit the *E. coli* RNase P holoenzyme.

Table 14.1 Molecules that have been used to target RNase P.

Molecule	Type	Target	Effect on RNase P	References
LNA 14-mer	Antisense	*E. coli* RPR	Inhibition ($IC_{50} = 3.9$ nM)	[13]
PNA 14-mer	Antisense	*E. coli* RPR	Inhibition ($IC_{50} = 12.5$ nM)	[13]
EGS^{Rpp38}	Antisense	HeLa RNase P (*in vivo*)	Reduction of Rpp38 mRNA and protein	[14]
Neomycin B	Aminoglycoside	*E. coli* RPR	Inhibition ($IC_{50} = 35 \pm 12\,\mu M$)	[15]
Neomycin B	Aminoglycoside	*E. coli* RNase P	Inhibition ($IC_{50} = 60 \pm 16\,\mu M$)	[15]
NeoR5	Aminoglycoside conjugate	*E. coli* RNase P	Inhibition ($IC_{50} = \sim 0.5\,\mu M$)	[16]
Neomycin B	Aminoglycoside	*D. discoideum* RNase P	Noncompetitive inhibition ($K_i = 143\,\mu M$)	[17]
Tobramycin	Aminoglycoside	*D. discoideum* RNase P	Noncompetitive inhibition ($K_i = 734\,\mu M$)	[17]
Gentamicin	Aminoglycoside	*D. discoideum* RNase P	Noncompetitive inhibition ($K_i = 1074\,\mu M$)	[17]
Kanamycin	Aminoglycoside	*D. discoideum* RNase P	Noncompetitive inhibition ($K_i = 1871\,\mu M$)	[17]
Paromomycin	Aminoglycoside	*D. discoideum* RNase P	Noncompetitive inhibition ($K_i = 1414\,\mu M$)	[17]
Puromycin	PPTase inhibitor	*D. discoideum* RNase P	Competitive inhibition ($K_i = 3.46$ mM)	[18]
Amicetin	PPTase inhibitor	*D. discoideum* RNase P	Noncompetitive inhibition ($K_i = 2.75$ mM)	[18]
Blasticidin S	PPTase inhibitor	*D. discoideum* RNase P	Noncompetitive inhibition ($K_i = 7.4$ mM)	[18]
2,2′-bis(4-Hydroxyphenyl)-6,6′-bis-benzimidazole	Bis-benzimidazoles	*E. coli* RPR	Inhibition ($IC_{50} = 5.3\,\mu M$)	[19]
T4MPyP	Porphyrin	*E. coli* RPR	Competitive inhibition ($K_i = 0.96\,\mu M$)	[20]
TMAP	Porphyrin	*E. coli* RPR	Noncompetitive inhibition ($K_i = 4.1\,\mu M$)	[20]
PPIX	Porphyrin	*E. coli* RPR	Competitive inhibition ($K_i = 1.9\,\mu M$)	[20]
Acitretin	Synthetic retinoid	*D. discoideum* RNase P	Competitive inhibition ($K_i = 8\,\mu M$)	[21]
SPM-ATRA conjugate 8	Spermine–retinoic acid conjugate	*D. discoideum* RNase P	Competitive inhibition ($K_i = 0.5\,\mu M$)	[22]
Ro 13-7410	Arotinoid	Keratinocyte RNase P	Competitive inhibition ($K_i = 37\,\mu M$)	[23]
Anthralin	Hydroxyanthrone	*D. discoideum* RNase P	Inhibition ($IC_{50} = 400\,\mu M$)	[24]
Calcipotriol	Vitamin D analog	*D. discoideum* RNase P	Bimodal action Activation (limiting $C = 50\,\mu M$) Inhibition ($IC_{50} = 180\,\mu M$)	[25]
Spiramycin	Macrolide	*E. coli* RPR/RNase P	Activation	[26]

Apart from the steric blocking of structural elements at *E. coli*, P RNA by antisense oligonucleotides, a different antisense approach targeting human RNase P's protein subunits, was used to inhibit RNase P activity. An external guide sequence (EGS) that binds to the mRNA encoding the Rpp38 protein subunit of the holoenzyme was designed to transfect HeLa cells [14]. According to this strategy, the EGS-target RNA complex formed a structure that resembles the pre-tRNA conformation; thus, the complex is recognized by endogenous RNase P holoenzyme and is cleaved successfully [27, 28]. Almost 24 h after transient transfection of HeLa cells with plasmid that encodes EGS^{Rpp38}, reduced expression was observed, both in the mRNA and the protein level. In addition, the expression of four other RNase P protein subunits (Rpp29, Rpp25, Rpp21, hpop5) was inhibited by the Rpp38 downregulation, implying a probable correlated regulation of their expression [14]. In a similar approach, EGSs and siRNA have been used to inhibit expression of Rpp21, Rpp25, and Rpp29 protein subunits from HeLa RNase P *in vivo*, revealing results similar to those reported for EGS^{Rpp38} [29]. In both studies, 24 h after transient transfection, the cell stops growing, indicating the inhibition of RNase P *in vivo* through the downregulation of the protein subunits' expression, while the RNase P activity is restored between 60 and 72 h after transfection because the cells start to lose the plasmid that encodes the EGS.

14.3 Aminoglycosides

Aminoglycoside antibiotics (Figure 14.2) are flexible, positively charged compounds that readily interact with various RNA molecules, displaying a wide array of effects. When bound to ribosomes, they cause misreading of the genetic code through binding to a conserved sequence on the A-site of 16S rRNA in the 30S ribosomal subunit. They induce a conformational change in the rRNA, which causes incorporation of noncognate aminoacyl-tRNA (aa-tRNAs) and, therefore, the translational fidelity is lowered [30, 31]. On the other hand, aminoglycosides interact with other RNA molecules, mainly ribozymes (M1 RNA, hammerhead, hairpin, hepatitis delta virus (HDV), group I intron), through a different mechanism. Studies on aminoglycoside inhibition of human HDV, hammerhead ribozyme, self-splicing group I intron, and RNase P from several organisms [32, 33], suggest that these antibiotics suppress enzyme activity by replacing essential magnesium ions with protonated amino groups. Similar results were obtained after studying the aminoglycosides' effect on nuclear RNase P activity from the slime mold *Dictyostelium discoideum* [17] and human epidermal keratinocytes [34]. Kinetic analysis showed that inhibition of nuclear RNase P by aminoglycosides is Mg^{2+} dependent. Neomycin, tobramycin, gentamicin, kanamycin, and paromomycin behave as classical noncompetitive inhibitors, with neomycin being the strongest [17]. More specifically, inhibition of RNase P cleavage by neomycin B is pH dependent, indicating that there is a

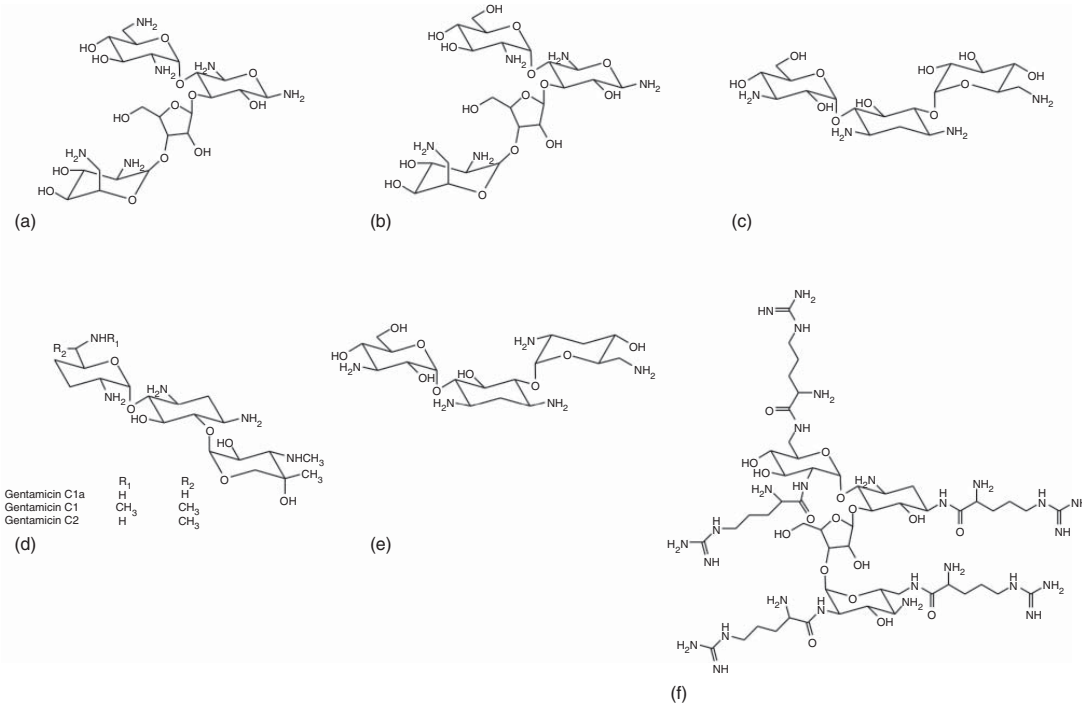

Figure 14.2 Structures of aminoglycosides: (a) neomycin B, (b) paromomycin, (c) tobramycin, (d) gentamicin, (e) kanamycin, and (f) aminoglycoside-derivative NeoR5.

direct relationship between the available positively charged amino groups in these antibiotics and their inhibitory potential [15, 17].

On the basis of these, an effort to improve the inhibitory potency of aminoglycosides on bacterial RNase P activity was undertaken with the synthesis of a series of aminoglycoside conjugates with arginine (R), guanidinium (G), and lysyl (K) residues [16]. In particular, NeoR5 (penta-arginine derivative of neomycin B) (Figure 14.2) was the most potent inhibitor having an IC_{50} 0.5 μM. Under identical assay conditions, both NeoK6 and NeoG6 were 10-fold weaker, while neomycin B was 800-fold weaker than NeoR5. Moreover, NeoR5 showed a different effectiveness on archaeal RNase P; type A RNase P (catalytically active RNase P RNA) was inhibited quite significantly, while type M RNase P (inactive RNase P RNA) was modestly activated [16, 35]. It was concluded that the inhibitor's potency depends on the molecular backbone, as well as the length, flexibility, and composition of the side chains. In addition, arginine aminoglycoside conjugates seem to inhibit bacterial RNase P more effectively than eukaryal RNase P. It has been suggested that neomycin B binds to the P-15 loop of the *E. coli* RNase P RNA subunit (M1 RNA, type A) (Figure 14.1) in such a way that it displaces a Mg^{2+} ion, which is probably involved in the chemistry of the cleavage. Mutations in the *E. coli* RPR's L15 loop, which weaken Mg^{2+} binding to L15, resulted in a threefold increase in the IC_{50} for neomycin B [15]. This result is partially corroborated by the effect of NeoR5 on archaeal RNase P activity [16]. On the other hand, NeoR5 inhibits the *E. coli* RNase P L15/P16/P17 mutant as effectively as it inhibits the wild-type RNase P [16], suggesting that the explanation for the phenomenon might be more complex than it was initially proposed.

14.4 Peptidyltransferase Inhibitors

Peptidyl transferase inhibitors such as puromycin, amicetin, and blasticidin S (Figure 14.3) also inhibit RNase P activity. As mentioned, puromycin, a mimic of the 3′ terminal end of the aminoacyl-tRNA, was the first inhibitor of RNase P activity reported [36]. Puromycin, amicetin, and blasticidin S do not act as slow-binding inhibitors, as in the ribosome complex. Detailed kinetic analysis established their modes of inhibition as simple competitive in the case of puromycin and as noncompetitive for amicetin and blasticidin S. It was also shown that amicetin and blasticidin S do not have additive inhibitory effects, suggesting that these compounds compete for binding on a common site. On the basis of the comparison of K_i values of the three molecules, amicetin is the stronger inhibitor [18, 37]. Other peptidyl transferase inhibitors such as chloramphenicol, spiramycin, and lincomycin, which act on prokaryotic ribosomes, as well as anisomycin that acts on eukaryotic ribosomes, have been tested in a similar way. However, they did not show any effect on RNase P activity under the conditions tested [37].

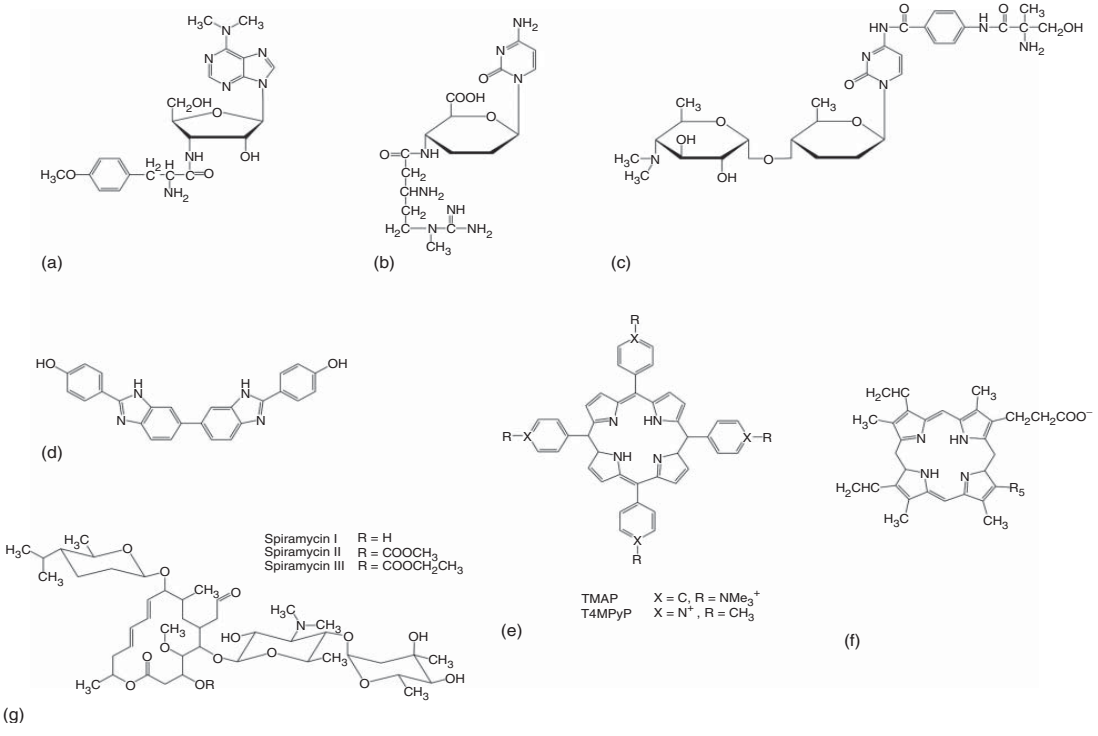

Figure 14.3 Structures of (a) puromycin, (b) amicetin, (c) blasticidin S, (d) porphyrins (T4MPyP, TMAP), (e) protoporphyrin IX, (f) bis-benzamidazoles (2,2′-bis(4-hydroxyphenyl)-6,6′-bis-benzimidazole), and (g) spiramycin.

14.5
Substrate Masking by Synthetic Inhibitors

A synthetic compound that belongs to benzimidazoles can bind to the T stem of *E. coli* $tRNA^{Phe}$ [38], a region implicated in tRNA binding to RNase P. Subsequently, a set of fully synthetic bis-benzimidazoles (Figure 14.3) were tested for their effect on P RNA-mediated catalysis and, indeed, several of these compounds inhibited pre-tRNA processing by *E. coli* M1RNA. The calculated IC_{50} values, between 5 and 21 μM, make these compounds among the strongest known inhibitors of RNase P, and the first fully synthetic inhibitors [19]. It seems that those compounds act at least partly on the substrate, as inferred from the fact that they perturbed the interaction of T- and D-loop with dissociation constants from low micromolar to the high nanomolar range, and they confer to substrate masking.

Along the same line, and motivated by evidence that cationic porphyrins bind to tRNA at sites important for tRNA tertiary structure formation and possibly P RNA binding, the effect of additional compounds that inhibit RNase P activity such as porphins and porphyrins on the cleavage reaction by *E. coli* P RNA was analyzed [20]. With K_i values of 0.8–4.1 μM, the porphyrins T4MPyP (*meso*-tetrakis(*N*-methylpyridyl) porphine), TMAP (*meso*-tetrakis[4-(trimethylammonio)phenyl] porphine), and PPIX (protoporphyrin IX) (Figure 14.3) are among the strongest small ligand inhibitors of the RNase P reaction described so far. Fluorescence data indicated that 1 : 1 complexes of these compounds form with *E. coli* P RNA. As these compounds bind with similar affinity to P RNA (K_ds of around 50 nM) and ptRNAs or tRNAs (K_ds of 0.1–1.2 μM), inhibition may be based on compound binding to P RNA, to substrate, or to both. It has also been suggested that a possible mechanism includes blockage of substrate–P RNA interaction, displacement of crucial metal ions, and induction of conformational changes within RNA.

14.6
Peculiar Behavior of Macrolides on Bacterial RNase P

Ribozyme activators are rarely described, but one could notice that there have been no significant efforts toward this direction. Several macrolides tested at concentrations of up to 1 mM did not show any effect on eukaryotic RNase P from *D. discoideum* [37]. In contrast, the macrolides spiramycin (Figure 14.3), erythromycin, tylosin, and roxithromycin affected the *E. coli* holoenzyme and M1 RNA-alone reaction in the low micromolar range, where they acted as dose-dependent activators [26]. Detailed analysis of the activation by spiramycin revealed a mixed-type activation mode with, at saturating concentrations of spiramycin, an 18-fold increase of k_{cat}/K_s in the holoenzyme reaction and a 12-fold one in the RNA-alone reaction. In the pH range from 5 to 9, the activation was shown to be pH independent, possibly indicating the involvement of hydrophobic interactions in the binding of the macrolide to M1 RNA. Also, binding of spiramycin on the M1 RNA does not alter the ribozyme's dependence on Mg^{2+} (Mg^{2+} concentration between

10 and 100 mM). The evidence regarding the precise mode of spiramycin-mediated activation of RNase P was supported by two different approaches: (i) kinetic analysis demonstrated that spiramycin affects the catalytic step of the reaction in the RNA-alone as well as the holoenzyme reaction and (ii) according to primer extension analysis data, binding of spiramycin to *E. coli* P RNA leads to a structural rearrangement of the P10/11 region, as shown after foot-printing analysis. In this region, A124, which appears conserved in bacterial P RNAs but not in eukaryotes, becomes more exposed, thereby providing a possible explanation for the insensitivity of *D. discoideum* RNase P to the stimulatory effect of macrolides. This may lead to increased affinity for the substrate and/or facilitated product release [26].

14.7 Antipsoriatic Compounds

The observation that retinoids, both natural (retinol and all-*trans* retinoic acid) as well as synthetic (isotretinoin and acitretin) can inhibit RNase P from *D. discoideum* was quite unexpected [21]. Retinoids represent a group of natural and synthetic analogs of vitamin A with important and pleiotropic effects on cell proliferation and differentiation and are used as drugs of choice for a wide spectrum of severe and recalcitrant skin disorders, such as psoriasis [39].

The initial report on the inhibition of a eukaryotic RNase P by retinoids was quite intriguing, as it was known that retinoids could propagate their intracellular signal through binding to nuclear receptors, indicating that, at least *in vitro*, alternative molecular target could account for retinoids and presumably RNase P was among those targets. That report was also supportive of preexisting (albeit overlooked) studies indicating that retinoids could trigger pleiotropic intracellular effects, and that their mode of action did not necessarily involve interaction with receptors. In that study, it was shown that all retinoids tested (both natural: all-*trans* retinoic acid and retinol; and synthetic: isotretinoin and acitretin) could effectively inhibit RNase P in a simple competitive way. Among retinoids, acitretin (Figure 14.4) was proved the most potent inhibitor of *D. discoideum* RNase P.

At the same time, the search for new retinoids that are more potent but also less toxic led to the development of the third retinoid generation, the arotinoids. Although all-trans retinoic acid, isotretinoin and acitretin, are stronger inhibitors than arotinoids [21, 40], the compound Ro 13-7410 (Figure 14.4) showed the strongest inhibitory effect on human epidermal keratinocyte RNase P among all vitamin A analogs [23]. Moreover, it has been suggested that these compounds may bind to allosteric inhibition sites of the enzyme.

The efficacy of calcipotriol (Figure 14.4), a synthetic analog of vitamin D_3, in the topical treatment of psoriasis and other keratinization disorders, has been established in a large number of clinical trials. As in the case of retinoids, calcipotriol can bind RNase P and modulates the activity of the enzyme from *D. discoideum* through a bimodal mode of action [25]. This biochemical behavior

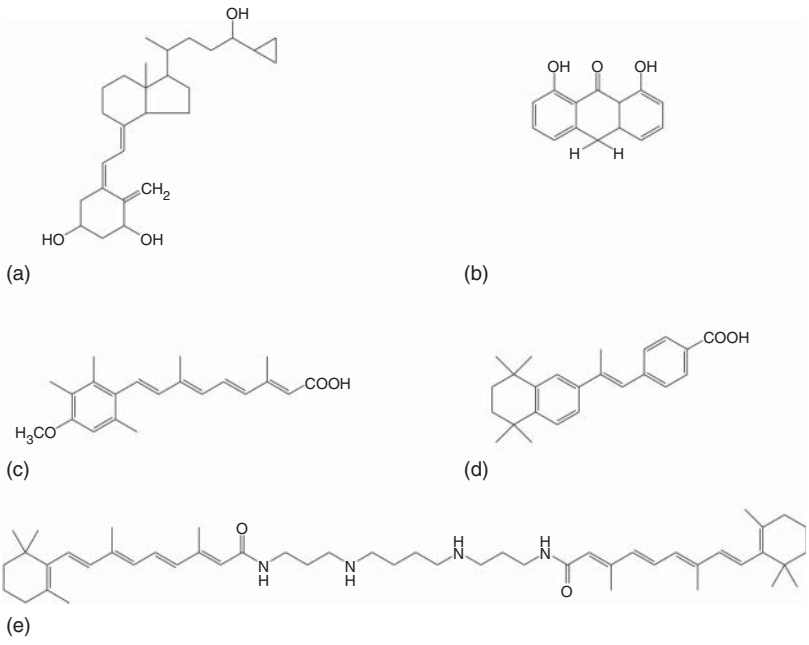

Figure 14.4 Structures of (a) calcipotriol, (b) anthralin, (c) acitretin, (d) Ro 13-7410, and (e) ATRA-SPM conjugate 8, the most potent inhibitor among spermine conjugates with acidic retinoids on RNase P activity [22].

suggests an apparent ambivalent (activating/inhibitory) effect possibly due to a multisite interaction of calcipotriol with RNase P. Moreover, anthralin (dithranol, 1,8-dihydroxy-anthrone) (Figure 14.4), which has been widely used for many years, for the treatment of chronic plaque psoriasis, also affects tRNA biogenesis *in vitro* causing a dose-dependent inhibition of *D. discoideum* RNase P [24] through a mechanism similar to calcipotriol.

The effects of calcipotriol and anthralin on *D. discoideum* RNase P were investigated separately or in combination, and interestingly, it was observed that the inhibition was additive [41]. This interesting effect provided the experimental basis and rationale for a therapeutic application in the management of psoriasis that included the combination of both compounds. In a clinical study, carried out subsequently, this notion was verified. The efficacy and safety of short-contact treatment with anthralin ointment (2%) versus its combination with calcipotriol ointment were comparatively investigated in two groups of patients with chronic plaque psoriasis [42]. The advantage of the combination therapy was already evident by the end of the first week of treatment and gradually became more pronounced in the subsequent weeks. By the end of the fifth week of the trial, all patients treated with the combination regimen revealed a complete remission of their plaques, whereas the patients treated with short-contact anthralin alone, even after 6 weeks, still exhibited psoriatic lesions with a considerable Psoriasis Area Severity Index (PASI) score even after 6 weeks of therapy (1.21 ± 1.00).

On the basis of all the accumulated biochemical data, a series of novel mono- and diacylated spermines, readily obtained using isolable succinimidyl active esters of acidic retinoids for the selective acylation of free spermine or *in situ* activated acidic retinoids for acylating selectively protected spermine, followed by deprotection. These novel compounds were designed on the basis of the action of their precursor compounds that were already effective on RNase P. These novel compounds (Figure 14.4) were shown to inhibit RNase P more strongly than their parent retinoids, following the prediction that was dictated by their chemical structure, thus opening the way for rational design of novel antipsoriatic compounds [22]. In addition, this new line of synthetic retinoic acid–spermine conjugates are more effective, they also exhibit antitumor properties when tested in cell cultures [43] and most importantly, they do not burden any clinical trials with undesired side effects, such as teratogenesis and aberrant cell proliferation, when tested in rats (D. Drainas, unpublished data). All these suggest that a thorough and in-depth investigation of eukaryotic RNase P function can lead to the design of novel, more effective, and less toxic compounds, with a plurality of effects on major cellular events.

14.8 Conclusions and Future Perspectives

RNase P represents an essential enzyme that changed our view of molecular evolution and became the first biochemical evidence for the existence of the ''RNA world.'' Today, almost 40 years after its discovery, it still intrigues as it holds an important position in the transition to the RNP world. It is more than evident that apart from the bacterial ribozyme, which has been extensively studied, eukaryotic RNase P can be used as a suitable molecular target. It is also evident that RNase P resembles the ribosome and can be used for similar studies toward the understanding of the drugs' modes of action, and, more importantly, for the design of drugs specifically targeting RNase P. All the above-mentioned data clearly show that several drastic compounds, apart from their well-known suppressive action on protein and DNA synthesis and their regulatory effects on transcription, are also capable of directly affecting tRNA biogenesis by inhibiting RNase P activity through mechanisms that, however, require further investigation. RNase P represents a reliable biological target and a screening system for many compounds that might be helpful in the selection and clinical application of new and more potent agents and in the better understanding of the mode of their therapeutic action.

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15
Involvement of Ribosome Biogenesis in Antibiotic Function, Acquired Resistance, and Future Opportunities in Drug Discovery

Gloria M. Culver and Jason P. Rife

15.1
Introduction

Ribosomes are responsible for cellular protein synthesis. While the general architecture is the same for all ribosomes, regardless of origin, there are enough critical differences that allow some ribosome inhibitors to therapeutically target pathogenic ribosomes over human ribosomes. In considering drug selectivity, we have to consider three ribosome forms: ribosomes of the pathogen, human cytoplasmic ribosomes, and human mitochondrial ribosomes.

The ability to selectively target bacterial ribosomes over human ribosomes has been a mainstay of modern antibiotic chemotherapy, where a subset of clinical antibiotics target ribosome function, usually by either causing miscoding or preventing peptidyltransferase action (Figure 15.1) [1]. As with virtually all clinically used antibiotics, their use has been marginalized with the expansion of antibiotic resistance. Therefore, many companies and academic laboratories are focused on enhancing the antibiotic arsenal by a variety of means including discovering new targets, reforming existing antibiotics, and shutting down specific mechanisms of resistance.

Selectively shutting down ribosome biogenesis in bacteria is a concept that has been addressed to some degree [2–4]. Some antibiotic resistance mechanisms chemically alter the bacterial ribosome, a special condition of ribosome biogenesis, rendering them insensitive to some antibiotics. Therapeutically shutting down these resistance mechanisms would restore sensitivity to these same antibiotics [5, 6]. A third concept to be addressed in this review is the observation that pathogens can be rendered avirulent or less virulent by deletion of a ribosome biogenesis factor, which has implications for both vaccine development and chemotherapeutic intervention of infections.

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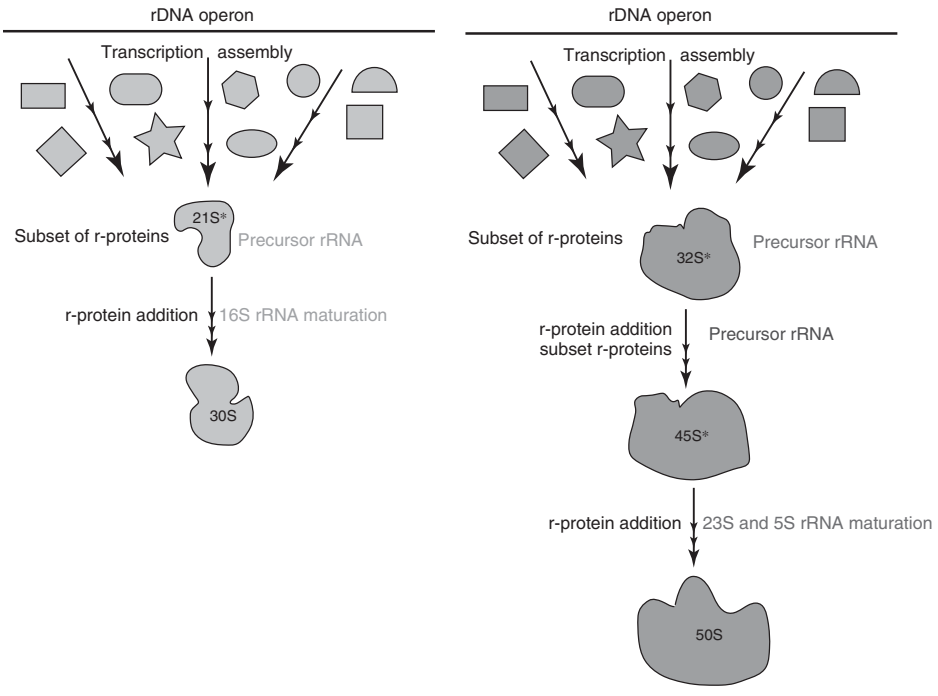

Figure 15.1 Illustration of ribosome biogenesis in *E. coli* and commonly observed bottlenecks. Disruption of ribosome biogenesis factors or growth under otherwise suboptimal conditions leads to the build up of a set of often seen intermediates. General compositions of these intermediates and their relative positions within the biogenesis pathway are indicated.

15.2 Ribosome Biogenesis

Ribosome biogenesis, regardless of the system, involves certain broad features including the synthesis of overly long pre-rRNA transcripts that must be trimmed down to mature lengths by nucleases, the modification of a small number of ribosomal RNA (rRNA) nucleotides and ribosomal protein amino acids, and the use of chaperones to aid in the refolding of rRNA and integration of ribosomal proteins. In all organisms, these broad steps appear to occur in a partially concurrent manner, the specific details appear to be deeply differentiated at the phylogenetic level. As details and specifics of the eukaryotic and prokaryotic ribosome biogenesis pathways are revealed, putative antimicrobial targets suitable for therapeutic intervention will be uncovered.

Ribosome biogenesis in bacteria has been most extensively studied in *Escherichia coli*, with a few studies in other bacteria offering a slightly broader depth [7, 8]. Even with limited knowledge of the *in vivo* cascade that results in the production of mature bacteria ribosomal subunits, it is clear that new opportunities for drug

development are available in the biogenesis pathway [3]. While for many years the bacterial ribosomal subunit assembly was simply examined as a process involving mature rRNA sequences and ribosomal proteins (r-proteins), recent work has shed light on the complexity of this system. A small number, relative to the process of eukaryotic ribosome synthesis, of extraribosomal biogenesis proteins have been identified in bacteria and an appreciation of the role that modification enzymes (rRNA and r-protein) can play in biogenesis has emerged. Thus, a critical role for a variety of biogenesis factors, precursor rRNA sequences, and their processing and modification machineries and the interplay of these factors and components has now been established or proposed in earnest for bacterial systems. The hypothesis that obstructing the biogenesis of bacterial ribosomal subunit could lead to effective therapeutics or changes in virulence has thus taken root.

While both eukaryotes and prokaryotes follow a similar overall program for ribosome synthesis, many of the details and players appear to be distinct. While the individual loss of several *E. coli* ribosome biogenesis factors results in very mild phenotypes, there are several that have profound effects on growth (see [9, 10] for examples). Moreover, several of these are conserved throughout pathogenic bacteria but not in host organisms [3]. These findings strengthen the likelihood of identifying appropriate novel drug targets that are part of pathogenic bacterial ribosome biogenesis pathways. However, for many identified biogenesis factors, a definitive and specific function in maturation of bacterial ribosomal subunits has not been clearly established. Thus, the field awaits the identification of a clear and appropriate auxiliary factor target for drug discovery.

15.3
Antibiotics and Ribosome Biogenesis

Numerous antibiotics that either cause miscoding in translation or inhibit peptide bond formation have been reported to also prevent the maturation of a small percentage of ribosomal precursors *in vivo* (Figure 15.1). The aminoglycosides paromomycin, neomycin [11], and hygromycin B [12] have been shown to alter small subunit assembly (Figure 15.2). It was initially reported that erythromycin and azithromycin could inhibit large subunit assembly [13, 14]. Subsequently, it has been demonstrated that erythromycin and chloramphenicol produce defects in assembly of both the large and small ribosomal subunits [15], and changes in assembly of both subunits have now been reported in cells treated with neomycin [16]. Detailed investigation of the mode of subunit assembly inhibition points to a change in ribosomal protein synthesis, resulting in an imbalance that apparently alters biogenesis for at least some of these antibiotics [15]. While the blocks to ribosomal subunit biogenesis have not been delineated in each case, it is clear that treatment with these antibiotics alters the synthesis of ribosomal subunits and could thus contribute to the negative effect that these molecules have on bacterial growth.

One challenge in studying bacterial ribosome biogenesis is the apparent redundancy in the system. *In vitro* work has indicated that there are multiple, parallel

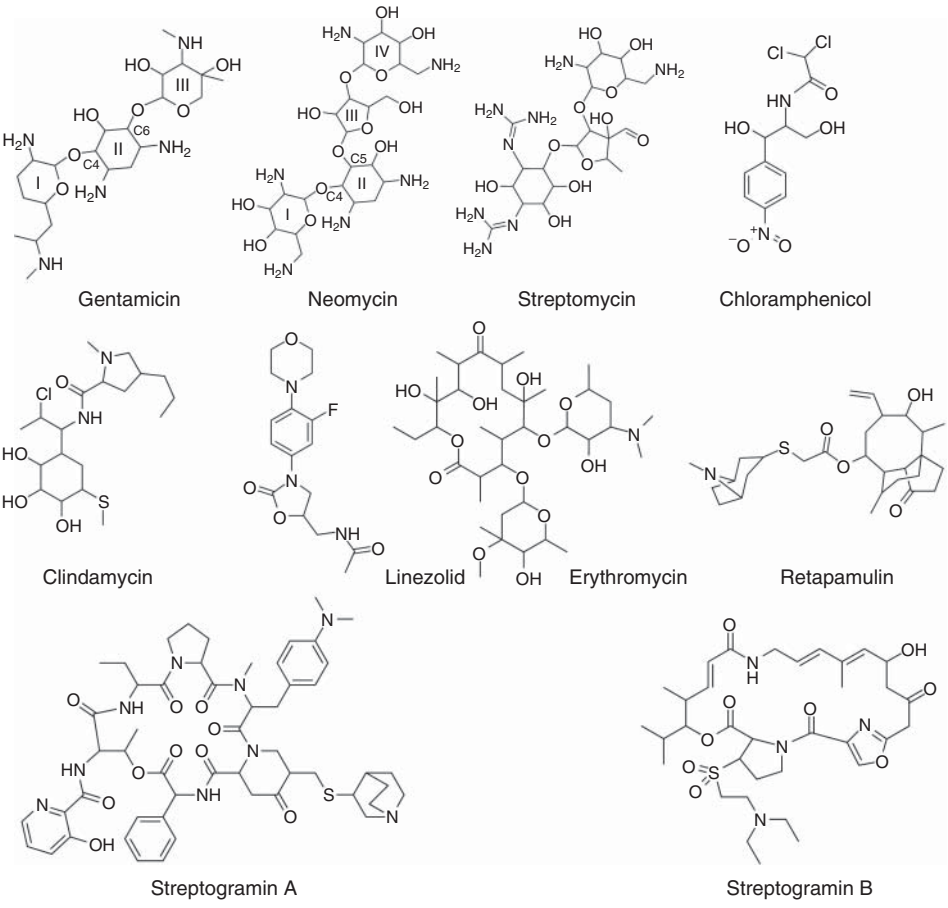

Figure 15.2 Chemical structures of antibiotics discussed in this chapter. Each agent is a representative of a unique class of clinically available antibiotics that both targets the ribosome and can be rendered ineffective with an acquired resistance rRNA methyltransferase. Two subclasses of aminoglycoside antibiotics are defined by either a 4,5 linkage or a 4,6 linkage at ring II, as noted in the structures of gentamicin and neomycin.

pathways that can result in the formation of 30S subunits [17–19]. In addition, deletion of genes for ribosomal subunit biogenesis factors can lead to subtle phenotypes (see [7, 8] for an overview). Also, many of these mutations that alter biogenesis result in similar changes in ribosomal subunit profiles (see [20–22]). For example, many mutations that alter small ribosomal subunit assembly produce limiting but detectable amounts of particles that sediment near 21S (see [22] for example). These 21S particles are also similar to those produced upon subunit maturation defects associated with antibiotic treatment (see preceding text). Thus, many different conditions or insults lead to the same output in the small subunit biogenesis cascade; these conditions include changes in 16S rRNA sequence (see [20]),

mutations in ribosomal proteins [22], deletion ribosome biogenesis factors (see [21]) and antibiotic treatment [23]. These findings suggest that the multiple pathways that appear to occur in parallel early in the cascade converge on a single bottleneck that must be resolved for biogenesis to continue. A similar conclusion can be made for the pathway of large subunit assembly. Many different mutations result in the formation of two large subunit intermediates, one that sediments at approximately 32S and the second that sediments near 45S (see [24]). Again, treatment with antibiotics that inhibit large subunit assembly also results in the production of similar intermediates (see [15]). Thus, it appears that assembly of the two subunits follows a similar theme with many parallel, engaged steps that converge at a common point (or two) and that resolution of these bottlenecks is critical for efficient ribosomal subunit biogenesis (Figure 15.1). Moreover, some work demonstrates (see [16, 22]) that the same conditions or treatment can block biogenesis of both subunits, suggesting that there may be common targets that could be especially important for drug design. Thus, understanding the nature of the bottlenecks and how they are resolved *in vivo* will be critical for identifying new antimicrobial targets and drugs.

15.4 Methyltransferases

The state of nucleotide methylation can alter the ribosome's sensitivity to many antibiotics (Figure 15.3). There are, generally speaking, two classes of rRNA methyltransferases in bacteria responsible for nucleotide methylation. The first

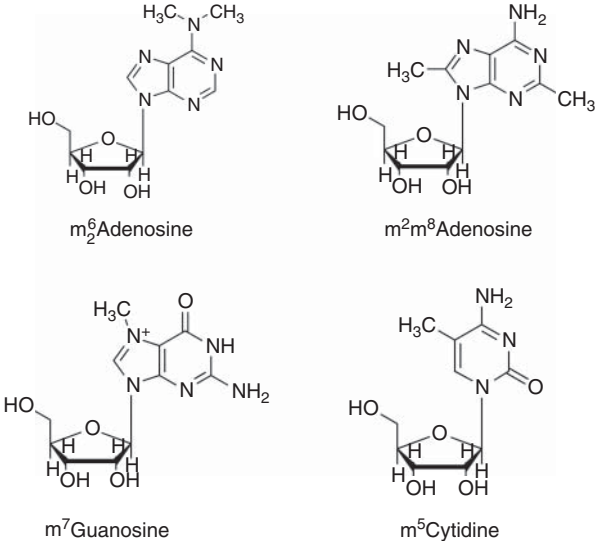

Figure 15.3 Some types of methylated nucleotides found in bacterial rRNA. These specific nucleotides where chosen because they correspond to the ones addressed in Table 15.1 and Figure 15.4.

group has been referred to as *housekeeping methyltransferases* as they are part of the normal cadre of ribosome biogenesis factors and are always present and active when ribosomes are being made. Housekeeping rRNA methyltransferases account for approximately 50% of the known total of ribosome biogenesis factors in *E. coli* (see [7]).

A second type of rRNA methyltransferases is specifically related to antibiotic resistance. Most or all of this second type coevolved with the production of antibiotics in producing strains of bacteria [25]. Over time, under selective pressure they have been disseminated to other bacteria including many human pathogens. Most notably, some classes of antibiotic resistance methyltransferase limit the clinical utility of several aminoglycoside antibiotics, macrolide, lincosamide, and streptogramin B (MLS_B) antibiotics, and phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A $PhLOPS_A$ antibiotics (Table 15.1; Figure 15.2). These methyltransferases alter the chemical structure of the ribosome at the sites where antibiotics bind with the consequence of reducing drug affinity (Figure 15.4) [26–29]. The reason so many classes of antibiotics can be inactivated by only a few types of rRNA methyltransferases is because most clinically useful antibiotics bind to a handful of ribosomal sites [1].

On the whole, both housekeeping rRNA methyltransferases and especially resistance rRNA methyltransferases have strong links to antibiotic function. While rRNA methyltransferases can be reasonably assigned to one of the two groups, there are a few examples where classification is ambiguous and probably reflects the evolutionary transition of a housekeeping methyltransferase into a resistance methyltransferase or vice versa. The deep evolutionary roots between natural antibiotics and rRNA methyltransferases has yielded a situation where most clinically relevant ribosome-acting antibiotics can be rendered useless in the presence of the right rRNA methyltransferase. A few examples are presented here to illustrate common concepts.

KsgA was the first housekeeping rRNA methyltransferase to be discovered by virtue of its relationship to kasugamycin, a natural product primarily used to treat rice blast [30]. KsgA converts two adjacent adenosines in 16S rRNA (A1518 and A1519, *E. coli* numbering) into N^6,N^6-dimethyladenosines by utilizing *S*-adenosylmethionine (SAM) as a methyl donor [31]. It has been shown in the laboratory setting that several species of bacteria can evolve resistance to kasugamycin through mutation of the *ksgA* gene with the consequence that A1518 and A1519 are no longer dimethylated [30, 32–35]. It has been recently observed that deletion of the *ksgA* gene in both *E. coli* and *Staphylococcus aureus* renders the organisms slightly more sensitive to some clinical aminoglycosides, including gentamicin (O'Farrell, H. and Rife, J.P., unpublished results) [36]. Interestingly, both effects, resistance to kasugamycin and sensitivity to gentamicin, are indirect as neither A1518 nor A1519 form part of the binding pocket of either kasugamycin or gentamicin [37, 38].

The existence of ErmC, a structural paralog of KsgA, was coincidently discovered the same year as KsgA (1971) and represents the first member of the resistance class of rRNA methyltransferases [26]. Similar to KsgA, it also catalyzes the

Table 15.1 Ribosome-binding antibiotic classes used clinically and corresponding resistance methyltransferases.

Drug	Methyltransferase	Modification site	Stage of biogenesis	Remarks
			Small subunit (30S)	
Gentamicin (4,6 class of aminoglycosides)	ArmA/Rmt	m^7G1405	Late	ArmA/Rmt and NpmA provide high-level protection to AB. Absence of KsgA provides low-level resistance.
	NpmA	m^1G1408	Late	
	EfmM	m^5C1404	Late	Presence of EfmM provides low-level resistance in *Enterococcus faecium*
	KsgA	$m^6{}_2A1518$ $m^6{}_2A1519$	Late	
Neomycin (4,5 class of aminoglycosides)	NpmA	m^1G1408	Late	NpmA provides high-level protection to aminoglycoside AB. Presence of EfmM provides low-level resistance in *Enterococcus faecium*
	EfmM	m^5C1404	Late	
	RsmG (GidB)	m^7G527	Early/intermediate	Deletion of rsmG confers high resistance in *Salmonella*
Spectinomycin	None	—	—	—
Streptomycin	RsmG (GidB)	m^7G527	Early/intermediate	Delection of *rsmG* yields low-level resistance and potentiates the probability of *rspL* mutation leading to high-level resistance; deletion of rsmG confers high resistance in *Salmonella*
Tetracycline	None	—	—	—

(*continued overleaf*)

Table 15.1 (*Continued*)

Drug	Methyltransferase	Modification site	Stage of biogenesis	Remarks
			Large subunit (50S)	
Chloramphenicol	Cfr	m^2m^8A2503[a]	Early	AB is a member of the $PhLOPS_A$ grouping of antibiotics
Clindamycin[b] (lincosamide)	Erm Cfr	$m^6{}_2A2058$ m^2m^8A2503	Early/intermediate Early	AB is part of both of the MLS_B and $PhLOPS_A$ groupings of antibiotics
Erythromycin (macrolide)	Erm	$m^6{}_2A2058$	Early/intermediate	AB is part of the MLS_B grouping of antibiotics
Fusidic acid	None	—	—	—
Linezolid	Cfr	m^2m^8A2503	Early	AB is a member of the $PhLOPS_A$ grouping of antibiotics
Retapamulin	Cfr	m^2m^8A2503	Early	AB is a member of the $PhLOPS_A$ grouping of antibiotics
Streptogramin A	Cfr	m^2m^8A2503	Early	AB is a member of the $PhLOPS_A$ grouping of antibiotics
Streptogramin B	Erm	$m^6{}_2A2058$	Early/intermediate	AB is part of the MLS_B grouping of antibiotics

[a] A2503 is first converted to $m^8$2503 by the housekeeping methyltransferase RlmN. This modified nucleotide serves as the substrate for Cfr.
[b] Some forms of Erm only monomethylate, a condition that is sufficient to confer resistance to the lincosamides.

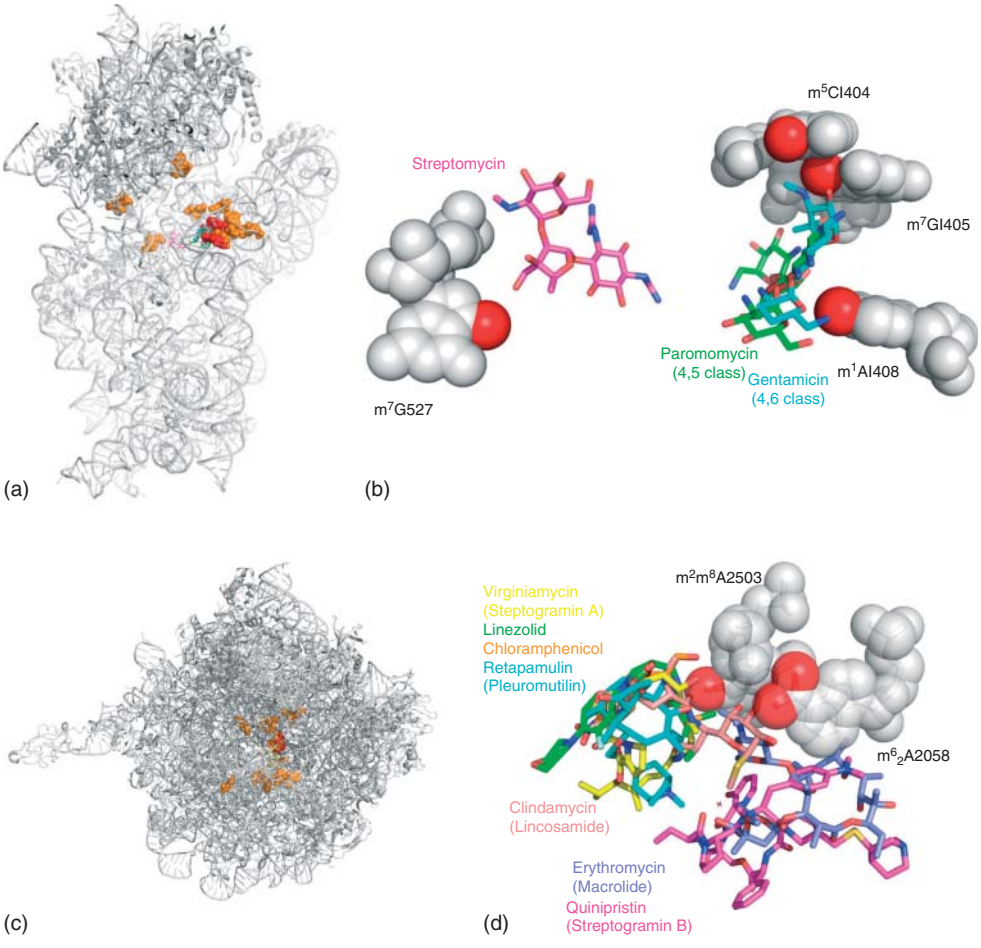

Figure 15.4 Location of methylated nucleotides and binding sites of antibiotics within the subunits of *E. coli*. In (a) (30S subunit) and (c) (50S subunit) the modifications that result from housekeeping are indicated in orange, while those acquired resistance methyltransferases are indicated in red. (b) (30S subunit) and (d) (50S subunit) show the interaction between numerous antibiotics and nucleotides that confer high-level resistance either when methylated or when typical methylation is lost (G527). Methyl groups are shown in red.

conversion of adenosine into N^6,N^6-dimethyladenosine, which is not surprising given the close structural relationship between the two enzymes [39]. However, in this case, ErmC modifies A2058 of 23S rRNA (*E. coli* numbering). Thus, the principal functional difference between KsgA and ErmC lies in their abilities to recognize different ribosomal substrates. ErmC is one of many related Erm proteins that confer high-level resistance to MLS_B classes of antibiotics [40]. The so-called MLS_B antibiotics are chemically distinct antibiotics but share a common binding site and resistance mechanism (Erm). As a group, the Erm methyltransferases

constitute an exceedingly important group of resistance enzymes because of their wide distribution throughout many bacterial pathogens, and when present render useless the clinically critical class of macrolide antibiotics and other second-tier antibiotics.

ArmA/Rmt (m^7G1405) and NpmA (m^1A1408) are two distinct classes of resistance methyltransferases that confer high-level resistance to clinically important aminoglycoside antibiotics by modifying specific residues in 16S rRNA of the small ribosomal subunit [29, 41]. Unfortunately, as with *erm* resistance genes, these amingoglycoside resistance genes have made substantial inroads into numerous pathogenic bacteria [28].

The Cfr methyltransferase makes m^2m^8A2503 from m^8A2503 of 23S rRNA and in doing so confers resistance to several chemically distinct classes of antibiotics referred to as *Phenicols, Lincosamides, Oxazolidinones, Pleuromutilins, and Streptogramin A* [42, 43]. The housekeeping methyltransferase RlmN also methylates this same nucleotide at the 2-position and shows a clear evolutionary relationship to Cfr [42, 43]. Cfr and RlmN are part of a unique radical-SAM methyltransferase family that utilize two SAM molecules and are more properly referred to as a *methyl synthases* rather than methyl transferase and operate by a mechanism distinct from most all other SAM-dependent methyltransferases [44, 45].

Numerous methyltransferases are hard to classify as either housekeeping or antibiotic resistance methyltransferases. For example, we generally think of Erm enzymes as being of the latter type. However, many mycobacteria species, including *Mycobacterium tuberculosis*, are intrinsically resistant to macrolide antibiotics, in part, because they encode for Erm enzymes from ancient chromosomal genes [46]. Other housekeeping rRNA methyltransferases that are known to correlate cellular sensitivity to antibiotics are RsmG and EfmM. RsmG, widely distributed throughout eubacteria, is responsible for forming m^7G527, and when absent in *Streptomyces coelicolor*, *M. tuberculosis*, and *Bacillus subtilis* makes ribosomes somewhat less sensitive to the aminoglycoside streptomycin [47–49]. Evidence suggests that *rsmG* mutation might be a clinically relevant mechanism of streptomycin resistance in *M. tuberculosis* [50]. Mutation of *rsmG* and the consequent modest streptomycin resistance increases the likelihood of high-level streptomycin resistance via a second mutation in the *rspL*, the gene that encodes for the ribosomal protein S12 [48]. In a *Salmonella* species, deletion of *rsmG* was reported to confer high-level resistance to both streptomycin and neomycin [51]. EfmM provides modest resistance to aminoglycoside antibiotics through formation of m^5C1404 in *Enterococcus faecium* [52].

15.5 Methyltransferase Integration into the Ribosome Biogenesis Pathway

rRNA methyltransferases, including those from the antibiotic resistance class, can be very selective about the form of the substrates they recognize. For example, Erm enzymes can methylate isolated 23S rRNA, small fragments from rRNA, but not

mature 50S subunits [53, 54]. Erythromycin can indirectly induce the formation of a pre-50S particle, which can serve as a substrate for ErmC [15, 55]. From these observations it is clear that Erm methyltransferases do not act on a fully mature 50S particle, but rather must integrate into the ribosome biogenesis pathway during an intermediate assembly time point. Given that *erm* genes of one form or another have been acquired by numerous divergent bacterial pathogens, the constraints of integration must not be too stringent or the mechanism ribosome biogenesis is well conserved throughout eubacteria.

On the other hand, Sgm and RmtC, members of the ArmA/Rmt family of aminoglycoside methyltransferases, do not seamlessly integrate into 30S subunit biogenesis in *E. coli*. Expression of either Sgm or RmtC leads to an undermethylation of m^5C1407, a product of the housekeeping methyltransferase RsmF [56, 57]. This observation suggests that within the temporal path of ribosome biogenesis, the ArmA/Rmt methyltransferases act before (RsmF) does even though mature 30S subunits function as substrates *in vitro*. Despite reduced methylation by RsmF, there is no apparent fitness cost to *E. coli* growth rate when RmtC is expressed. In fact, lack of RsmF alone reduced aminoglycoside susceptibility for *E. coli*, illustrating both direct and indirect effects RmtC has on aminoglycoside resistance. A late-stage pre-30S particle from *E. coli* serves as an efficient substrate for ArmA [36]. RsmG is unique among the small subunit methyltransferases involved in antibiotic resistance in that it methylates early in the ribosome biogenesis pathway [58], whereas all others are late acting [29, 52, 59, 60]. The relative time points for methyltransferase action during ribosome biogenesis are summarized in Table 15.1.

A final note is that the ability of resistance and housekeeping methyltransferases to integrate in highly divergent organisms is not universal. The aminoglycoside resistance genes *sgm* and *kgmB*, when expressed in *S. cerevisiae*, a eukaryotic organism, failed to modify rRNA in spite of the strong sequence conservation found in the decoding region of small subunit rRNA among all domains of life [61].

15.6 Ribosome Biogenesis Factors, Virulence, and Vaccine Development

There is a growing body of evidence indicating that the lack of a functional ribosome biogenesis factor in bacteria can profoundly reduce the virulence of a pathogen. Several years ago, it was observed that a *ksgA* deletion strain of *Yersinia pseudotuberculosis*, a pathogen that causes disease in animals and occasionally in humans, was avirulent to mice when challenged with it [62]. Subsequent to that study, it was shown that this same avirulent strain could act as an attenuated pathogen with the ability to serve as a vaccine [63]. Mice immunized with a *ksgA* deletion strain showed protection rates of either 91 or 100%, depending on the number of cells of parental *Y. pseudotuberculosis* used in the challenge.

Strains of *Erwinia amylovora* rendered resistant to the atypical aminoglycoside kasugamycin, by virtue of mutations within the *ksgA* gene, showed reduced virulence against pear fruit, a natural host [35]. Counterintuitively, the observed

lower virulence rate could not be correlated with aberrant growth rates within either the fruit itself or in artificial liquid medium. Therefore, it would appear that reduced virulence in at least this case is related to specific virulence factors, perhaps their underexpression.

A third example where lack of functional KsgA affects infectivity at the organismal or cellular level was shown with the sexually transmitted disease pathogen *Chlamydia trachomatis*, a bacterium that invades host mammalian cells [64]. The authors of this report noted that small plaques of mouse fibroblast cells formed with a *ksgA* deletion strain relative to parental *C. trachomatis*. As there is no animal model for this pathogen, there is no way to state that lack of active KsgA affects virulence, but the reduced rate of host cell death is consistent with observations noted earlier for *Y. pseudotuberculosis* and *E. amylovora*.

The absence of at least one other ribosome biogenesis factor can lead to an avirulent strain of pathogenic bacterium. Knockout of the *rsgA* (*yjeQ*), a small ribosomal subunit ribosome GTPase, in *S. aureus* leads to an avirulent strain in a mouse kidney abscess infection model [65]. In this case, the *rsgA* deletion strain showed reduced growth rate in liquid medium and in the kidney, which coincided with reduced weight loss and superior physical appearance and alertness in mice infected with the mutant strain relative to those infected with the parental strain. From these observations the authors suggest that RsgA is an attractive drug target. RsgA is also a good candidate drug target as it is a P-loop GTPase [66].

At present, it is too early to know whether targeting KsgA or other ribosome biogenesis factors is a viable route to reducing the virulence of pathogenic bacteria as a direct treatment of infection or as a means of generating attenuated strains for vaccination, but some lessons are already evident. First, it is an overly simplistic analysis to dismiss a potential drug target on the basis of bacterial growth rates in standard liquid and agar media because such rates do not necessarily correlate with infectivity efficiency. Second, in the case of *E. amylovora* we suggest that there appears to be a link between the presence of active KsgA and specific mechanism of virulence, although this connection has not been determined. Finally, much more work needs to be done in assessing the importance of ribosome biogenesis and opportunities for therapeutic intervention. KsgA illustrates this point well. In the three examples of KsgA presented here, the discoveries that KsgA is important to virulence and infectivity were all serendipitous. ''Essentiality'' has been a defining quality when screening for novel antibiotic drug targets in the post-genomic era [67]. This is usually taken to mean that a given gene cannot be successfully knocked out. We suggest that this is perhaps too stringent a definition. After all, in 3–4 billion years of evolution, life has not seen fit to do without KsgA outside the laboratory setting, despite it not being ''essential'' in any known bacterial organism. Understanding that evolution is not sentimental, KsgA's retention in all bacteria must be for important reasons, as illustrated earlier.

Without the development of new therapies to treat bacterial infections, the twenty-first century will begin to look more like the premodern era of antibiotics – a time when people succumbed to the diseases of bacterial infections above all other maladies. Unfortunately, the strategy of identifying essential genes through

knock-out and subjecting those protein targets to vast chemical libraries in high-throughput screening assays has been ''disappointingly unsuccessful for antibiotic research'' [67]. Lessons from the genomic-wide drug discovery efforts prominent in the first decade of this millennium suggest that targets should be naturally complex in function, antiresistance mechanisms to permit the use of current drug classes are important, and that novel strategies such as addressing virulence should be pursued. Also, identified targets should be ubiquitous to permit a broad spectrum of activity. Ought not ribosome biogenesis to become a larger subject of study in antibiotic drug discovery?

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16
Aminoacyl-tRNA Synthetase Inhibitors

Urs A. Ochsner and Thale C. Jarvis

16.1
Introduction

The alarming increase in incidence of infections caused by drug-resistant bacteria has created an urgent need for new antibacterial agents. Most drugs currently in development are derivatives of members of known antibiotic classes, raising the likelihood that resistance will emerge quickly upon widespread use. Overcoming resistance is a primary consideration in new antibacterial development, and compounds with a novel mechanism of action offer attractive advantages. The molecular target of new antibacterial drugs determines many key functional properties including spectrum of activity, mode of action, rate of resistance emergence, and toxicity potential.

The aminoacyl-tRNA synthetases (aaRSs) represent a large class of essential enzymes that catalyze the charging of tRNAs with their cognate amino acids, synthesizing essential precursors needed by ribosomes to translate RNA into protein. Thought to be among the first enzymes to evolve during the transition from a primordial RNA world [1, 2], these enzymes are found in all kingdoms of life and play an integral role in translation of the genetic code. Given their essential role in protein synthesis, the aaRS family represents a large group of potential antibacterial targets. In fact, a number of naturally occurring compounds that inhibit aaRS activity selectively have been identified, with concomitant antibacterial activity. Mupirocin, a natural product that inhibits isoleucyl-tRNA synthetase (IleRS), is the only aaRS inhibitor that is currently approved as an antibiotic. Thus, the aaRS family offers a rich variety of currently underexploited antibacterial targets.

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16.2
Enzymatic Mechanism of Action of aaRS

16.2.1
Condensation of Amino Acid and Cognate tRNA

Most organisms encode 20 aaRSs, one for each amino acid. Specific aaRSs are typically referred to by their three-letter amino acid code followed by "RS" (e.g., leucyl-tRNA synthetase is abbreviated LeuRS).[1)] aaRSs catalyze the 3′ esterification of a tRNA to its cognate amino acid, producing an aminoacyl-tRNA (also known as a *charged* tRNA). The enzymatic mechanism of aaRSs has been extensively studied, and enzymatic studies are further supported by three-dimensional structures of many aaRSs [3–6]. Some aaRSs associate in multienzyme complexes that facilitate efficient interaction with related cellular processes, as reviewed by Hausmann *et al.* [7].

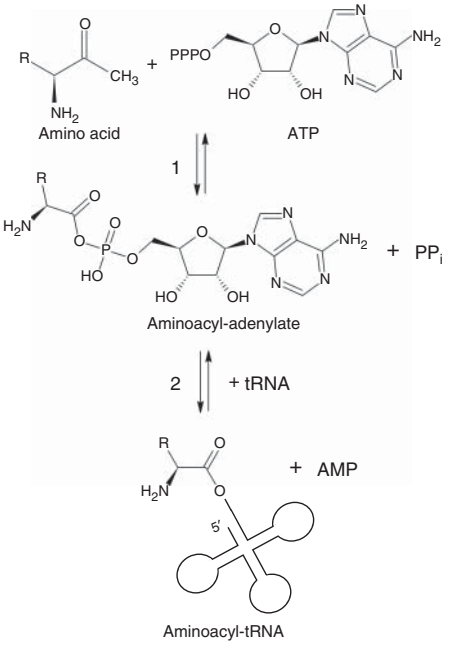


Figure 16.1 aaRS reaction mechanism. aaRSs catalyze charging of their cognate tRNAs in two steps. In the first step, the enzyme binds the amino acid and ATP in the active site. This positions the α-carboxylate of the amino acid correctly for inline nucleophilic attack at the α-phosphate of ATP, forming the mixed anhydride (aminoacyl-adenylate) intermediate, with release of inorganic pyrophosphate. In the second step, the 2′-OH or 3′-OH from the 3′-terminal nucleotide of the cognate tRNA acts as the nucleophile to attack the α-carbonyl of the aminoacyl-adenylate, resulting in 3′-esterification of the tRNA and release of adenosine monophosphate (AMP).

1) The standard nomenclature for tRNAs is to refer to uncharged tRNA as, for example, $tRNA^{Leu}$ or leucine tRNA, while the aminoacylated (charged) tRNA is denoted as leucyl-tRNA or Leu-tRNA.

Most synthetases catalyze the formation of the aminoacyl-tRNA product via a two-step reaction (Figure 16.1). In the first step, the enzyme binds the amino acid and ATP and catalyzes a condensation reaction to yield the aminoacyl adenylate intermediate with release of pyrophosphate. In the second step, the enzyme catalyzes transfer of the aminoacyl group from the adenylate to the 3′-end of the tRNA (either to the 2′ OH or the 3′ OH, depending on the specific aaRS), with release of adenosine monophosphate (AMP).

16.2.2 Classification of aaRS

Despite sharing a common reaction mechanism, the aaRSs fall into two completely distinct structural classes (Table 16.1). The differences center on the architecture of the active site and the mode of binding to tRNA. Class I enzymes have a Rossman dinucleotide-binding fold, made up of alternating β-strands and α-helices. At the primary sequence level, class I enzymes are recognizable on the basis of two signature motifs, one being a 12-amino-acid sequence ending in HIGH and a second KMSKS pentapeptide motif [5, 8]. These sequence elements form crucial structures at the site of adenylate synthesis. In class I synthetases, the Rossman fold is divided by an insertion called the *connective polypeptide* 1 (*CP*1), which, in a subset of cases, contains another active site that is involved in correcting misactivated amino acids. The active site of class II aaRSs is composed of three

Table 16.1 Classification of aminoacyl-tRNA synthetases.

Class I			Class II		
Ia	**Ib**	**Ic**	**IIa**	**IIb**	**IIc**
ArgRS	GlnRS	TrpRS	GlyRS[a]	AsnRS	AlaRS
CysRS	GluRS	TyrRS	HisRS	AspRS	GlyRS[a]
IleRS			ProRS	LysRS-II[b]	PheRS
LeuRS			ThrRS		
MetRS			SerRS		
ValRS					
LysRS-I[b]					
Active site: Rossman fold			Active site: seven-stranded β-sheet		
Sequence motifs: KMSKS, HIGH			Sequence motifs: Motif 1–3		
ATP conformation: extended			ATP conformation: bent		
Aminoacylation regiospecificity: 2′-OH			Aminoacylation regiospecificity: 3′-OH		
tRNA orientation: aaRS contacts minor groove and variable loop faces solvent			tRNA orientation: aaRS contacts major groove and variable loop faces aaRS		

AaRSs are grouped into subclasses based on common structural and mechanistic properties [6, 5, 11].
[a]Two distinct forms of GlyRS have been observed in different species.
[b]LysRS is a class I enzyme in archaea and a few bacteria and class II in most other bacteria and in eukaryotes [12].

α-helices and an antiparallel seven-stranded β-sheet, characterized by three highly degenerate primary sequence motifs [8]. The two classes differ in regiospecificity; class I enzymes catalyze attachment of the amino acid to the 2′-OH of the RNA, while class II enzymes (with the exception of PheRS) attach the amino acid via the 3′-OH of the RNA. Crystallographic studies have revealed additional structural signatures differentiating the two classes, as shown in Figure 16.2 [9, 10]. Class I enzymes access the tRNA acceptor stem from the minor groove side, whereas class II enzymes utilize a mirror-image approach, accessing the tRNA acceptor stem from the major groove. In addition, most class II enzymes bind ATP in an unusual bent conformation, while class I enzymes bind ATP in the more common extended conformation [5]. The two classes of aaRSs appear to have arisen very early in evolution, and only LysRS has orthologs from both classes.

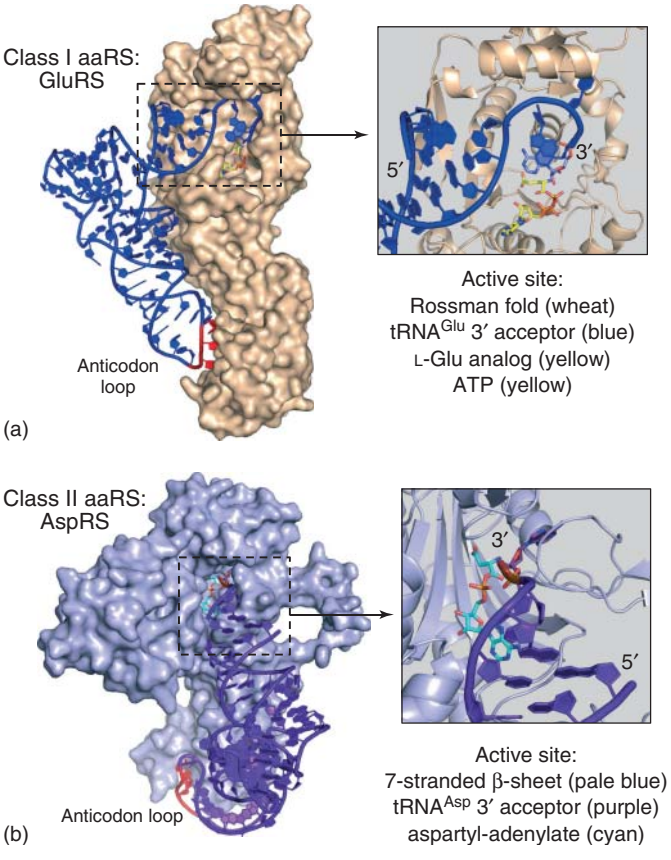


Figure 16.2 Examples of class I versus class II aaRS–tRNA complexes. (a) Quaternary complex of a class I aaRS: GluRS from *T. thermophilus* bound to $tRNA^{Glu}$, ATP, and an L-glutamine analog (PDB code: 2CV1) [10]. (b) Ternary complex of a class II aaRS: AspRS from *E. coli* bound to $tRNA^{Asp}$, and aspartyl-adenylate (PDB code: 1C0A) [9]. Anticodon residues are highlighted in red in each panel.

Although aaRSs fall into two general structural classes, they exhibit wide structural and mechanistic variations within each class. They have been grouped in subclasses based on various criteria, including type of amino acid charged (i.e., nonpolar vs charged), multimeric state (monomer, dimer, or tetramer), primary sequence similarity, whether or not tRNA binding is required during the adenylation step, and whether or not an editing domain is present [5, 13, 11]. Subclass Ia enzymes typically charge amino acids that are nonpolar/aliphatic or contain sulfur, and most have an editing domain. Although ArgRS lacks an editing domain and binds a charged amino acid, it is shown in subclass Ia (Table 16.1) based on primary sequence similarity. Similar to GlnRS and GluRS, ArgRS requires tRNA binding during the aminoacylation step, and for this reason ArgRS is sometimes grouped with subclass Ib [6]. TyrRS and TrpRS comprise subclass Ic; both bind aromatic amino acids and are obligate dimers. Subclass IIa enzymes are dimeric, bind amino acids that are aliphatic or polar, and have acylation sites composed of mixed β-sheets. Subclass IIb aaRSs are also dimeric, but bind charged or polar amino acids, and utilize an oligosaccharide-binding (OB) fold for the aminoacylation active site. Subclass Ic enzymes are tetrameric and bind nonpolar amino acids. There are two distinct forms of GlyRS in different phylogenetic species, accounting for its presence in both the IIa and IIc subclasses.

16.2.3 Fidelity and Proof Reading

Translational fidelity relies on the accuracy of the aminoacylation pathway, matching the correct amino acid to its specific cognate tRNA. The overall error rate of aaRSs is estimated at less than 1 in 10 000 [6]. The fidelity of aminoacylation relies first on highly accurate recognition of both the amino acid and the cognate tRNA. The size and complexity of tRNAs make specific recognition by an aaRS a relatively straightforward task. Each aaRS recognizes its cognate tRNAs via one or more of the following elements: the discriminator base N73 and/or acceptor stem, and/or the anticodon loop [14]. This is accomplished primarily through distinct structural modules that are appended to the active site domain and provide for proper anticodon recognition. Specific discrimination between certain amino acids is considerably more challenging, given their small size and shared features. In a subset of aaRSs that face particularly challenging discrimination requirements, editing mechanisms have evolved. Two different editing mechanisms have been identified [15]. Pretransfer editing involves hydrolysis of incorrect aminoacyl-adenylate intermediates, while post-transfer editing involves hydrolysis of incorrectly aminoacylated tRNA and can be catalyzed by the aaRS, or by a separate tRNA deacylase. Size discrimination has been proposed to occur via a double-sieve model, wherein the adenylation active site excludes incorrect amino acids that are too large, but may allow smaller amino acids to slip through [16]. Then the editing site, which is typically too small for the cognate amino acid, can correct small misacylated products. About half of the aaRSs exhibit a distinctly identified editing domain, with a hydrolytic active site for amino acid editing [17].

In certain other enzymes, such as MetRS, it appears that the aminoacylation active site itself catalyzes editing of misacylated amino acids [18].

16.2.4 Transamidation Pathway

Genomic analysis has revealed that archaea and most bacteria do not possess GlnRS. Instead, these organisms utilize a nondiscriminating Glu-RS (ND-GluRS), which catalyzes synthesis of both the correctly aminoacylated Glu-tRNA^{Gln} and misacylated Glu-tRNA^{Gln}. This misacylated product is then modified to Gln-tRNA^{Gln} through a phosphorylation/transamidation reaction catalyzed by a tRNA-dependent amidotransferase (AdT) known in bacteria as GatCAB AdT [19]. Similarly, archaea and about half of all bacteria lack AsnRS, and synthesize correctly charged Asn-tRNA^{Asn} via a bienzymatic reaction catalyzed by ND-AspRS and GatCAB. These bienzymatic mechanisms in bacteria are facilitated by the formation of a transamidosome complex consisting of aaRS, tRNA, and GatCAB [20].

16.2.5 aaRSs as Targets for Antimicrobial Agents: General Modes of Inhibition

As essential components of the protein synthesis pathway in bacteria, aaRSs remain attractive targets for antibacterial drug development. Inhibition of aminoacylation represents the most direct approach, leading to depletion of cellular pools of charged amino acids and hence to cessation of protein synthesis. Inhibition of aminoacylation can be achieved either by blocking the formation of the aminoacyl-adenylate intermediate or by blocking the esterification of the tRNA.

Inhibition of aaRS editing offers another potential avenue for achieving an antibacterial effect. Blocking editing in an aaRS that is prone to misacylation would result in accumulation of incorrectly charged amino acids, culminating in deleterious protein synthesis and impaired function. Although it is unclear whether this mechanism would achieve the same level of potency as direct inhibition of aminoacylation, genetic and biochemical evidence supports this as a viable strategy for antibacterial development. For example, an *Escherichia coli* mutant encoding an editing-defective IleRS exhibits severely retarded growth [21]. Overexpression of *Bacillus subtilis* GluRS in *E. coli* triggers mischarging of tRNA^{Gln} with Glu, resulting in cell death [22].

The transamidation pathway for indirect synthesis of Asn-tRNA^{Asn} and Gln-tRNA^{Gln} appears to be largely confined to bacteria and archaea. Although recent evidence has shown that transamidation is utilized in mitochondria in some lower eukaryotes [23], it has not been observed in the eukaryotic cytosol [24]. Thus, the bacterial transamidation pathway may provide a unique set of antibacterial targets. Blocking transamidation would result in accumulation of mischarged Glu-tRNA^{Gln} and Asp-tRNA^{Asn}, causing abnormal protein synthesis in a manner similar to inhibition of aaRS editing.

Inhibition of aaRS activity leads to accumulation of uncharged tRNAs, a condition also found during amino acid starvation. Uncharged tRNAs bind to the ribosome and interfere with normal protein biosynthesis, triggering an RelA-mediated response known as the *stringent response* [25]. The stringent response results in elevated levels of guanosine tetraphosphates (ppGpp) and guanosine pentaphosphates (pppGpp), which inhibit RNA polymerase. Thus, inhibition of aaRS activity has the direct effect of inhibiting protein synthesis, while triggering an indirect inhibition of RNA synthesis. The synergeristic antagonism of two major cellular biosynthesis pathways reinforces the ability of aaRS inhibitors to induce bacteriostasis.

16.3 aaRS Inhibitors

16.3.1 Mupirocin, a Paradigm

Mupirocin (pseudomonic acid A) inhibits IleRS and is the only marketed antibiotic that targets the aaRS family (Figure 16.3). *Pseudomonas fluorescens* strains that produce this compound harbor a second, protective synthetase (IleRS-R2) that is not sensitive to mupirocin and exhibits eukaryotic features [26]. Mupirocin is active against a number of pathogens, including *Staphylococcus aureus*, coagulase-negative staphylococci, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Enterococcus faecium* (not *Enterococcus faecalis*), *Haemophilus influenzae*, *Moraxella catarrhalis*, and – *in vitro* only – pathogenic fungi, including dermatophytes [27].

For decades, topical formulations of mupirocin (Bactroban) have been used for the treatment of skin infections such as impetigo and secondarily infected traumatic skin lesions [28, 29], and for nasal decolonization to prevent methicillin-resistant *S. aureus* (MRSA) infections in MRSA carriers [30, 31]. Not surprisingly, high clinical use of this antibiotic has led to the emergence of mupirocin-resistant staphylococci [32]. Low-level mupirocin resistance (MIC = 8–256 $\mu g\,ml^{-1}$) is caused by point mutations within the *ileS* gene, and high-level mupirocin resistance (MIC > 256 $\mu g\,ml^{-1}$) is mediated through acquisition of the *mupA* gene that encodes a second, phylogenetically distinct IleRS [33]. The mupirocin concentration in marketed creams and ointments is 2% (20 000 $\mu g\,g^{-1}$), but the effective concentration at the site of infection may be substantially lower because of drug release and diffusion effects. Nonetheless, mupirocin remains a powerful topical antibiotic and has an excellent safety and efficacy profile, serving to validate the family of aaRSs as useful drug targets.

16.3.2 Old and New Compounds with aaRS Inhibitory Activity

Selective inhibition of microbial aaRSs has been an attractive strategy in the search for new antibiotics. Over the past few decades, many aaRS inhibitors have been

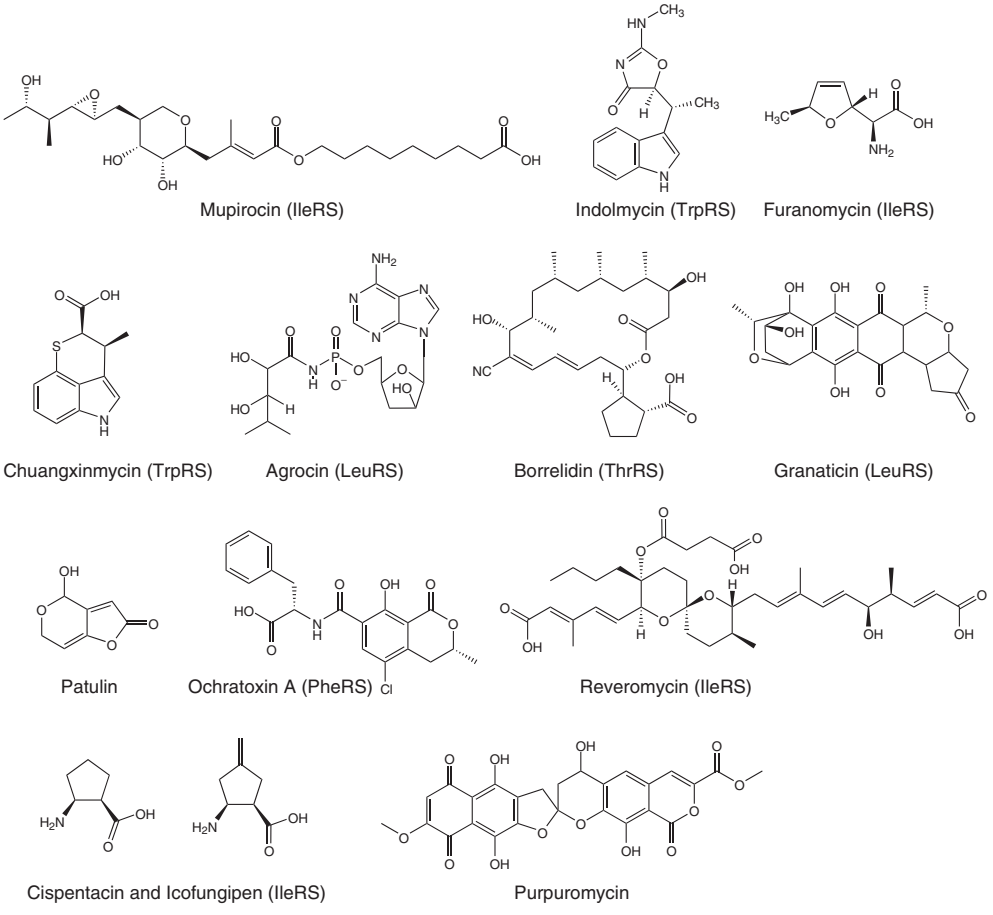


Figure 16.3 Natural compounds with aaRS inhibitory activity.

described, including a remarkable array of natural products and synthetic compounds that have been obtained by virtual screening and structure-based design, or via high-throughput biochemical screening of compound libraries [13, 34].

16.3.2.1 Natural Products That Inhibit aaRS

The existence of many other natural aaRS inhibitors beyond mupirocin validates these enzymes as attractive drug targets. Spanning an immense structural diversity (Figure 16.3), some of these compounds lack selectivity, have off-target activities, and show toxicity; thus, they have limited clinical use.

Indolmycin is produced by *Streptomyces griseus* and its antibacterial activity, particularly against *S. aureus*, was recognized over half a century ago [34]. Structurally similar to tryptophan, indolmycin inhibits both the tryptophan uptake pathway and the TrpRS aminoacylation reaction. Pfizer initiated clinical development of indolmycin in the 1960s, but abandoned the program owing to a narrow spectrum of activity

that excluded streptococci, enterococci, and Enterobacteriaceae, as well as liver toxicity related to tryptophan catabolism. More recently, indolmycin has been revisited by Takeda Pharmaceuticals (Osaka, Japan) as an agent (TAK-083) holding great promise against *Helicobacter pylori* TrpRS ($IC_{50} = 12.2$ nM, $MIC_{90} = 0.03\,\mu g\,ml^{-1}$). Preclinical data indicated bactericidal activity of TAK-083–which is rather unusual for aaRS inhibitors – that was maintained at low pH and efficacy in a gastric infection model with Mongolian gerbils, where complete clearance was achieved with a twice-daily regimen of $10\,mg\,kg^{-1}$ for 7 days [35]. Indolmycin has also been reconsidered as a topical agent to treat staphylococcal infections and MRSA nasal carriage, as it demonstrated excellent activity against methicillin-sensitive *S. aureus* (MSSA) ($MIC_{90} = 0.5\,\mu g\,ml^{-1}$), MRSA ($MIC_{90} = 1\,\mu g\,ml^{-1}$), vancomycin-intermediate *S. aureus* (VISA; MIC range, $0.12–2\,\mu g\,ml^{-1}$), and fusidic-acid- or mupirocin-resistant isolates ($MIC_{90} = 0.25\,\mu g\,ml^{-1}$); however, *in vitro* resistance to indolmycin appeared to develop readily [36].

A similar compound, also produced by certain *Streptomyces*, is the IleRS inhibitor furanomycin, which inhibits Ile-$tRNA^{Ile}$ formation by substituting for Ile in the charging of $tRNA^{Ile}$ in *E. coli* [37]. Chuangxinmycin is a natural TrpRS inhibitor produced by *Actinoplanes tsinanensis* ($IC_{50} = 30$ nM for the *E. coli* enzyme), and analogs synthesized at GlaxoSmithKline possessed moderate antibacterial activity, with $MIC = 4\,\mu g\,ml^{-1}$ against *S. aureus* and $MIC = 16\,\mu g\,ml^{-1}$ against *H. influenzae* and *M. catarrhalis* [38]. Agrocin, which harbors a 5′-phosphoramidate-bond-containing leucyl-adenylate analog that acts as a potent natural LeuRS inhibitor ($IC_{50} < 10$ nM), is used to inhibit the growth of *Agrobacter tumefaciens* strains that cause crown gall tumors in crops. This compound is produced by the nonpathogenic biocontrol agent *Agrobacterium radiobacter* K84; this strain contains a second, self-protective copy of the synthetase [39]. Microcin C, produced by some *E. coli* strains, has a similar molecular mode of action; it is a heptapeptide that contains a modified AMP covalently attached to the C-terminal aspartate. In the cell, microcin C degradation results in a modified aspartyl-adenylate containing an N-acylphosphoramidate linkage, which blocks AspRS [40]. Microcin C analogs containing different terminal amino acids of the heptapeptide (Asp, Glu, or Leu) attached to adenosine through a nonhydrolyzable sulfamoyl bond have been synthesized and were found to target AspRS, GluRS, and LeuRS [41]. Borrelidin, yet another *Streptomyces*-derived compound, is an 18-membered macrolide, and a known inhibitor of ThrRS. Strong antimicrobial and antiangiogenic properties as well as antimalaria activity have been reported [42, 43].

Several known toxins target aaRS, albeit not in a specific or selective manner [13]. Granaticin is an aromatic polyketide synthesized by *Streptomyces* that inhibits LeuRS. Patulin is a mycotoxin produced by a variety of molds, in particular *Aspergillus* and *Penicillium*, and it inhibits aminoacylation broadly and irreversibly. Ochratoxin-A is another mycotoxin isolated from *Aspergillus ochraceus* that inhibits PheRS of bacteria, yeast, and eukaryotes. The agent is immunosuppressive, teratogenic, and carcinogenic, possibly due to secondary modes of action that cause inhibition of phenylalanine hydroxylase, lipid peroxidation, and the formation of DNA adducts. The polyketide reveromycin A is a potent, yet nonselective inhibitor

of IleRS in yeast and higher eukaryotes and has attracted recent interest to develop novel agents to treat osteoporosis as well as osteolytic bone metastasis in lung cancer patients. The structurally related natural compounds spirofungin A and B that also inhibit IleRS in mammalian cells can be obtained via total synthesis and their antiproliferative activity has been demonstrated in a panel of cancer cell lines [44].

Cispentacin is a cyclic β-amino acid produced by *Bacillus cereus* with antifungal activity due to inhibition of IleRS. The compound demonstrated efficacy in the treatment of systemic infections with *Candida albicans* and *Cryptococcus neoformans*

Table 16.2 AaRS inhibitors identified through virtual screening or structure-based design, or by screening of compound libraries.

Screening/compound	Target (organism)	Activity (selectivity)	References
Benzthiazole derivative	MetRS (*S. aureus*)	$IC_{50} = 6.3\,\mu M$	[48]
Benzoic acid derivative	MetRS (*E. coli*)	$IC_{50} = 237\,nM$	[49]
3-aryl-4-arylaminofuranone	TyrRS (*S. aureus*)	$IC_{50} = 90\,nM$	[50]
Phenyl benzyl ether	PheRS (*H. influenza*)	$IC_{50} = 0.24\,\mu M$	[51]
Glutamyl-sulfamoyl-adenosine	GluRS (*E. coli*)	$K_i = 2.8\,nM$	[52]
Aspartyl-sulfamoyl-adenosine	AspRS (*E. coli*)	$K_i = 15\,nM$	[53]
Tyrosinyl adenylate	TyrRS (*S. aureus*)	$IC_{50} = 11\,nM$	[54]
Aminoalkyl-adenylate	ArgRS (*S. aureus*)	$IC_{50} = 7.5\,nM$	[55]
Aminoalkyl-adenylate	TyrRS (*S. aureus*)	$IC_{50} = 11\,nM$	[55]
Aminoacyl sulfamate	IleRS (*S. aureus*)	$IC_{50} = 4\,nM$	[55]
Aminoacyl sulfamate	ArgRS (*S. aureus*)	$IC_{50} = 4.5\,nM$	[55]
Isovanillate-hydroxamate	IleRS (*E. coli*)	$IC_{50} = 4.5\,\mu M$	[56]
Methionyl-adenylate analogs	MetRS (*E. coli*)	$IC_{50} = 0.4–2.4\,nM$	[57]
Aminoacyl-sulfamoyl thiazole	LeuRS (*E. coli*)	$IC_{50} < 2\,nM$	[58]
Aminoacyl-sulfamoyl thiazole	LeuRS (*S. aureus*)	$IC_{50} = 90\,nM$	[58]
SB-219383	TyrRS (*S. aureus*)	$IC_{50} = 1.4\,nM$ (22 μM, mammalian)	[59]
Bromo-thienyl ethanolamine	PheRS (*S. aureus*)	$IC_{50} = 8\,nM$	[60]
SB-425076	MetRS (*S. aureus*)	$IC_{50} = 1.4\,nM$ (> 1 μM, mammalian)	[61]
SB-203207	IleRS (*S. aureus*)	$IC_{50} = 1.7\,nM$ (< 2 nM, rat liver)	[62]
Phenyl-thiazolyl-sulfonamide	PheRS (*E. coli*)	$IC_{50} < 5\,nM$ (> 200 μM, mammalian)	[63]
2-Pyridyl-pyrazole derivative	MetRS (*S. aureus*)	$IC_{50} = 0.63\,\mu M$ (> 100 μM, human)	[64]
Spirocyclic furan	PheRS (*S. aureus*)	$IC_{50} = 2\,nM$ 30 nM (100 μM, human)	[65]
Heterocyclic inhibitor	PheRS (*S. aureus*)	$IC_{50} = 0.26\,\mu M$, $MIC = 3.1\,\mu g\,ml^{-1}$	[66]
Oxazolone-dipeptide	MetRS (*S. aureus*)	$IC_{50} = 18\,nM$ (> 100 μM, human)	[67]
Quinoline derivative	ProRS (*C. albicans*)	$IC_{50} = 5\,nM$ (> 20 μM, human)	[68]

in mice, without inducing acute lethal toxicity. A derivative, icofungipen (PLD-118, BAY 10–8888), had shown promising preclinical data and advanced as far as phase 2 clinical development by PLIVA, under license from Bayer, for the potential oral treatment of fungal infections [45, 46].

Purpuromycin, which is produced by *Actinoplanes ianthinogenes* and inhibits a broad range of bacteria and fungi, has a unique mode of action in that it binds to all tRNAs and inhibits their acceptor capacity by forming a nonproductive synthetase–tRNA complex [47].

16.3.2.2 **AaRS Inhibitors Identified in Screening Programs**

Potent aaRS inhibitors have been found via screening of compound libraries, using either an available aaRS crystal structure for virtual screening, or a functional biochemical assay to identify actual inhibitory compounds (Table 16.2, Figure 16.4).

Distinct structural classes of *S. aureus* MetRS inhibitors were identified in a virtual screen that was based on building a pharmacophore from a ligand-*S. aureus* MetRS structure and using this pharmacophore to screen a commercial

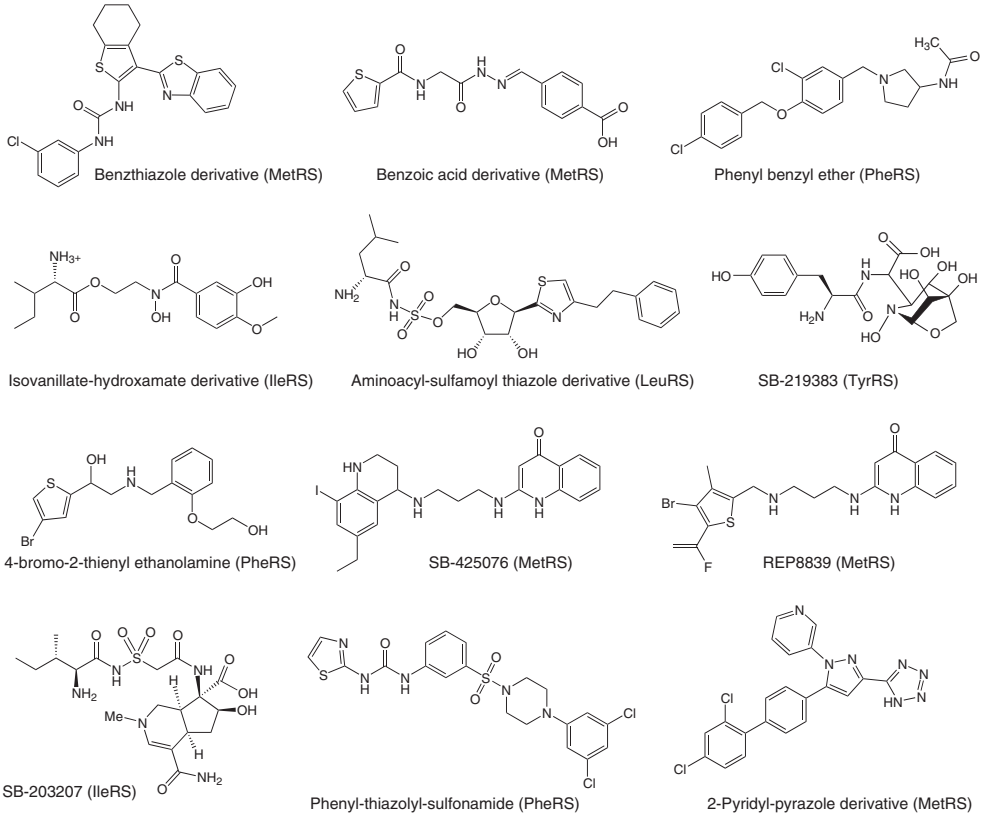


Figure 16.4 AaRS inhibitors identified by virtual or biochemical high-throughput screening.

database, followed by docking and testing the best screening hits; four of the compounds, including a benzthiazole derivative, proved to inhibit *S. aureus* MetRS with $IC_{50} < 10\,\mu M$ [48]. In a similar effort, a benzoic acid derivative that inhibited *E. coli* MetRS with $IC_{50} = 237\,nM$ was among 91 hits in a virtual screening of half a million compounds [49]. An interesting approach toward generating LeuRS inhibitors, described as virtual click chemistry, was based on the identification of key fragments for ligand binding within the catalytic pocket of LeuRS [69]. Other examples of virtual screening efforts focused on targets such as the parasite *Brugia malayi* AsnRS, the syphilis spirochete *Treponema pallidum* LysRS, and the Lyme disease agent *Borrelia burgdorferi* LysRS [13]. In recent molecular docking studies, 4-alkoxy-3-arylfuran-2(5H)-ones and 3-aryl-4-arylaminofuran-2(5H)-ones were identified as potent biochemical inhibitors of *S. aureus* TyrRS [50, 70].

AaRS inhibitors can be obtained from nonhydrolyzable substrate analogs (Table 16.2); however, such compounds often lack whole-cell activity. An IleRS inhibitor where isovanillate-hydroxamate [56] provides the surrogate moieties for adenine and ribose in isoleucyl adenylate, or a LeuRS inhibitor consisting of an aminoacyl-sulfamoyl thiazole [58] that mimics leucyl-adenylate are two of many such examples.

Screening campaigns conducted from 1995 to 2001 at SmithKline Beecham (GlaxoSmithKline) included all 19 aaRSs present in *S. aureus* but had only limited success [71, 72]. One such hit, SB-219383, a dipeptide composed of L-tyrosine and a novel bicyclic α-amino acid, had been purified from fermentation broth of *Micromonospora* sp. and demonstrated highly selective inhibition of bacterial versus mammalian TyrRS. Several series of semisynthetic derivatives of SB-219383 exhibited subnanomolar inhibitory activity against TyrRS, although *in vitro* activity against staphylococci and streptococci remained weak [59]. An interesting hit from the high-throughput screening effort directed at inhibitors of *S. aureus* PheRS was an ethanolamine derivative, which was further improved to 4-bromo-2-thienyl ethanolamine with $IC_{50} = 8\,nM$, but this compound also lacked activity against *S. aureus*, possibly due to efflux [60]. The *S. aureus* MetRS inhibitor screening program produced a potent initial hit ($IC_{50} = 350\,nM$), which was further optimized by synthesizing N,N-substituted derivatives of 1,3-diaminopropane, comprising phenyl, chroman, and tetrahydroquinoline subseries. The lead compound SB-425076 had low nanomolar potency, good antibacterial activity against staphylococci and enterococci, was highly selective over the mammalian counterpart, and demonstrated *in vivo* efficacy in an *S. aureus* rat abscess infection model [61, 73, 74]. This MetRS inhibitor series was out-licensed to Replidyne, Inc. in 2003, where further lead optimization resulted in REP8839, a compound that was developed as a topical agent to treat skin infections caused by *S. aureus* and *S. pyogenes* [75]. Phase 1 trials with REP8839 were successfully completed in 2007.

SB-203207, produced by *Streptomyces* and identified in a screening for IleRS inhibitors, had potent biochemical activity against the *S. aureus* enzyme but also against the eukaryotic enzyme from rat liver ($IC_{50} < 2\,nM$); in addition, weak off-target inhibition of LeuRS and ValRS was noted. Replacement of the Ile moiety with

Met and modifications of the bicyclic core yielded a somewhat more MetRS-specific inhibitor [62].

High-throughput screening of a synthetic compound library at Bayer identified a potent *E. coli* PheRS inhibitor [63]. The optimized compound, a phenyl-thiazolyl-sulfonamide, had low-nanomolar IC_{50}s for PheRS from *E. coli* and *H. influenzae* and was also active against *S. aureus* ($IC_{50} = 50–80$ nM). In contrast, it did not inhibit the mammalian cytoplasmic and mitochondrial PheRS ($IC_{50} > 200\,\mu M$). Kinetic measurements performed with *E. coli* PheRS showed that phenyl-thiazolyl-sulfonamides were competitive with Phe, but noncompetitive with ATP. *In vitro* antibacterial activity of the phenyl-thiazolyl-sulfonamide was demonstrated against the respiratory pathogens *S. aureus, S. pneumoniae, H. influenzae, and M. catarrhalis* ($MIC < 1\,\mu g\,ml^{-1}$), but *in vivo* efficacy in a murine *S. aureus* sepsis model was poor owing to substrate (Phe) antagonism.

Optimization of the 2-pyridyl-pyrazole lead compound identified via screening of a compound library resulted in potent *S. aureus* MetRS inhibitors (IC50 = 128 nM) with good selectivity over human MetRS; the mechanism of action, however, could not be attributed solely to inhibition of MetRS [64].

Similarly, screening for *S. aureus* and *E. coli* PheRS inhibitors identified a series of spirocyclic furans and related heterocyclic compounds; some analogs demonstrated antibacterial activity against *S. aureus* ($MIC = 3.1\,\mu g\,ml^{-1}$), but appeared to have a second, nonspecific mode of action in bacteria [65, 66].

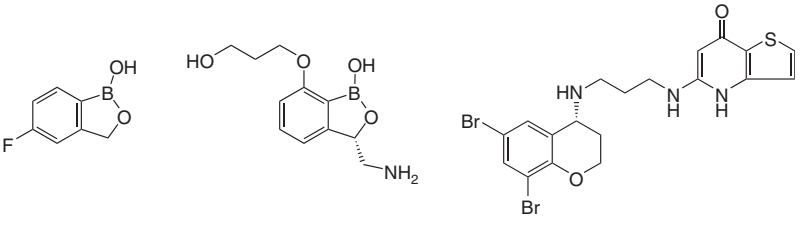
A phenyl benzyl ether was identified via screening for inhibitors of *H. influenzae* PheRS, but antimicrobial activity of the compound was affected by efflux [51].

Many other screening efforts yielded potent and selective biochemical inhibitors that unfortunately lacked antimicrobial whole-cell activity. Examples include a novel class of *S. aureus* MetRS inhibitors that contained an oxazolone-dipeptide scaffold [67], and quinoline inhibitors of *C. albicans* ProRS [68].

16.3.3 Novel aaRS Inhibitors in Clinical Development

16.3.3.1 CRS3123, a Fully Synthetic MetRS Inhibitor

CRS3123 (Figure 16.5) is a novel diaryldiamine inhibitor of bacterial MetRS currently in clinical development as an oral agent for treatment of *Clostridium*



AN2690 (tavaborole) AN3365 (GSK2251052) CRS3123 (REP3123)

Figure 16.5 Structures of aaRS inhibitors in clinical development.

difficile infection (CDI) [76]. CRS3123 (formerly known as REP3123) was derived from a compound series originally discovered through target-based screening at GlaxoSmithKline [73, 74]. The program was first acquired by Replidyne, and subsequently by Crestone.

CDI in the gastrointestinal tract causes symptoms ranging from severe diarrhea to toxic megacolon, and is associated with significant morbidity and mortality. Emergence of hypervirulent and drug-resistant strains of *C. difficile* has resulted in an alarming increase in incidence of CDI in the past decade, especially among elderly patients [77]. *C. difficile* is a spore-forming bacterium found in abundance in the environment and has the potential to colonize the gut, where it produces the toxins that cause CDI. Healthy normal gut flora keeps *C. difficile* growth in check; disruption of the gut flora due to exposure to broad-spectrum antibiotics predisposes the patient to CDI. Fidoxamicin and vancomycin are currently the only approved antibiotics for treatment of CDI, although metronidazole also receives considerable off-label use. Existing therapies are associated with high recurrence rates, often exceeding 20%, in particular with BI/NAP1/027 outbreak strains [78]. CRS3123 has exhibited potent microbiological activity against a large number of clinically relevant *C. difficile* strains. Its unique characteristics include limited potential for disruption of normal flora, inhibition of toxin production, and inhibition of sporulation in *C. difficile*. CRS3123 has low oral bioavailability in all species tested. Thus, oral administration of CRS3123 results in high concentrations of the drug in the gut and low systemic exposure.

CRS3123 exhibits potent antibacterial activity against all *C. difficile* strains tested ($N = 175$), including the epidemic BI/NAP1/027 strains, with MICs that range from 0.25 to $1\,\mu g\,ml^{-1}$ and an MIC_{90} of $1\,\mu g\,ml^{-1}$ [79, 80]. In contrast, CRS3123 has little or no activity against gram-negative anaerobes that are typically found in the intestine in large numbers such as Bacteroides spp., Prevotella spp., and *Porphyromonas asaccharolyticus* ($MIC_{90} \geq 32\,\mu g\,ml^{-1}$). The only exception is for selected strains of Fusobacterium spp., where all isolates are inhibited by CRS3123 at concentrations $\leq 1\,\mu g\,ml^{-1}$. Among gram-positive anaerobes, CRS3123 exhibits a greater selectivity against *C. difficile* when compared with vancomycin or metronidazole. CRS3123 is inactive against *Actinomyces* spp., *Bifidobacterium* spp., *L. casei/rhamnosus*, and *L. plantarum* with $MIC_{90}s \geq 32\,\mu g\,ml^{-1}$. The *in vitro* spectrum of activity of CRS3123 includes clinically important aerobic gram-positive cocci such as *S. aureus*, *S. pyogenes*, *E. faecalis*, and *E. faecium* ($MIC_{90}s < 1\,\mu g\,ml^{-1}$), but CRS3123 is generally not active against gram-negative bacteria.

As CRS3123 exerts its mode of action through the inhibition of protein synthesis, this agent exhibits desirable physiological effects on *C. difficile in vitro*, which may help in reducing the severity of disease and in limiting relapse and recurrence. Specifically, CRS3123 effectively inhibited toxin production in *C. difficile* cultures, where vancomycin or metronidazole had little effect [81]. Sporulation, which requires *de novo* synthesis of spore coat proteins, is suppressed even at sub-MIC

levels of CRS3123. In contrast, metronidazole and vancomycin promoted spore formation in some strains.

CRS3123 targets the MetRS subtype commonly found in gram-positive bacteria with a calculated inhibition constant (K_i) of 20 pM for *C. difficile* MetRS [79]. As such, it is one of the most potent aaRS inhibitors reported to date. Selectivity for the gram-positive MetRS subtype is very high; inhibition of human mitochondrial MetRS is >1000-fold weaker, and inhibition of human cytoplasmic MetRS is > 1 000 000-fold weaker. Inhibition of bacterial MetRS as the specific antibacterial mode of action of CRS3123 was demonstrated through target overexpression from a plasmid-borne, inducible copy of *met*S in *S. aureus*, which resulted in a 16-fold increase in the MIC for CRS3123. In macromolecular synthesis assays, CRS3123 exhibited a dose-dependent inhibition of protein and ribonucleic acid (RNA) synthesis in *S. pneumoniae* R6, but only protein synthesis was affected in an isogenic *rel* mutant deficient in the stringent response, as expected for an aaRS inhibitor.

Efficacy of CRS3123 has been evaluated in a hamster gastrointestinal infection treatment model [81]. Hamsters were pretreated with clindamycin to disrupt gut flora, followed by inoculation with *C. difficile*. CRS3123 exhibited superior efficacy to vancomycin, with 60–90% survival observed on day 28 as compared to 0–10% survival with vancomycin following twice-daily oral administration for 5 days at doses ranging from 0.5 to 5 mg kg^{-1}. Surviving animals treated with CRS3123 had healthy gross GI appearance and histopathology. In addition, low oral bioavailability was observed in healthy hamsters following oral administration, suggesting systemic exposure is not required to achieve efficacy.

16.3.3.2 AN2690 (Tavaborole) and AN3365 (GSK2251052), Boron-Containing LeuRS Inhibitors

Several benzoxaborole analogs that inhibit leucyl-tRNA synthetase (LeuRS) have been developed by Anacor, including AN2690 (tavaborole) and AN3365 (GSK2251052) shown in Figure 16.5. The precise mechanism of action has been determined for the benzoxaborole AN2690, which inhibits LeuRS by formation of a stable $tRNA^{Leu}$-AN2690 adduct in the editing site of the enzyme, via the boron atom of AN2690 and the 2′- and 3′-oxygen atoms of the 3′-terminal adenosine of the tRNA. The trapping of enzyme-bound $tRNA^{Leu}$ in the editing site prevents catalytic turnover, thus inhibiting synthesis of Leu-$tRNA^{Leu}$ and consequentially blocking protein synthesis [82].

Crystal structures have been obtained of the editing domains of human cytosolic LeuRS and *C. albicans* LeuRS. As a guide for SAR, the structure of the *C. albicans* LeuRS editing domain in complex with a related compound, AN3018, and AMP was determined at 2.2 Å resolution [83].

AN2690 (tavaborole) has broad-spectrum antifungal activity and is in development for the topical treatment of onychomycosis, a fungal infection of the toenails and fingernails [82, 84]. A particular challenge for any anti-onychomycosis agents is the poorly penetrable barrier of the nail plate. To be efficacious, topical antifungals

need to kill the causative fungi deep in the nail bed. Using an optimized formulation, AN2690 demonstrated good nail penetration and achieved levels within and under the nail plate that appear to be adequate for effective topical treatment for onychomycosis [85]. The onychomycosis market is substantial, with an estimated 35 million people in the United States alone who are affected by this condition. Current therapies for onychomycosis include debridement and drug therapies, with agents such as ciclopirox (Penlac) or terbinafine (Lamisil). Tavaborole demonstrated a safety and efficacy profile that could allow it to be a desirable therapy for the topical treatment of onychomycosis. Phase 1 and phase 2 clinical trials showed that tavaborole achieved significant nail penetration, resulted in little or no systemic exposure, and was well-tolerated. In three phase 2 clinical trials, tavaborole was efficacious, defined by achieving normal nail growth with absence of fungal elements on culture. The tavaborole phase 3 program consists of two double-blind, placebo-controlled trials with 600 patients in each arm, and enrollment started in late 2010. Two-thirds of the patients will receive tavaborole (5%), and one-third will receive vehicle once daily for 48 weeks. The primary endpoint is mycologic and clinical cure after 52 weeks [86].

GSK2251052 (AN3365) is also a member of the novel class of boron-containing antimicrobial protein synthesis inhibitors. Unlike a number of bacterial protein synthesis inhibitors, GSK2251052 does not inhibit mammalian mitochondrial protein synthesis *in vitro*, indicating a good selectivity profile [87]. GSK2251052 demonstrated excellent activity against Enterobacteriaceae ($MIC_{90} = 1\,\mu g\,ml^{-1}$, $n = 2029$), including strains with preexisting resistance mechanisms (extended spectrum β-lactamase, ESBL, *Klebsiella pneumoniae* Carbapenemase, KPC β-lactamases) and was also active against nonfermentative gram-negative bacilli including multidrug-resistant, MDR strains of *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophila*, and *Burkholderia cepacia* [87]. GSK2251052 showed $MIC_{90} = 4\,\mu g\,ml^{-1}$ against 2008 isolates of *P. aeruginosa* that were collected globally in 2009–2010. *In vitro* activity was also reported against a broad spectrum of anaerobic organisms. Furthermore, GSK2251052 was active against Category A and B bacterial biothreat agents including *Bacillus anthracis*, *Burkholderia pseudomallei*, *Francisella tularensis*, *Burkholderia mallei*, and *Yersinia pestis*, with $MIC_{90}s \leq 2\,\mu g\,ml^{-1}$ [87].

Early clinical development of the compound was initiated by Anacor, and a reassuring safety profile was demonstrated in phase 1. AN3365 (GSK2251052) was licensed to GlaxoSmithKline in July 2010. The agent was evaluated for the treatment of hospital-acquired gram-negative bacterial infections, particularly for difficult-to-treat infections caused by MDR organisms such as *P. aeruginosa*, but clinical development has been discontinued due to emergence of resistance. The *in vivo* efficacy of GSK2251052 has been demonstrated in a rat thigh suture model with challenge strains of *P. aeruginosa*, showing reductions of 2.9–4.2 $\log_{10}$ cfu/thigh compared to controls.

Distribution, metabolism, and excretion were studied in rats and monkeys following a single IV dose of ^{14}C-GSK2251052. The compound was widely and rapidly distributed to most tissues, and partitioning to blood cells was observed.

Significant quantities of an oxidative metabolite (M3) accumulated, whereby the hydroxypropyl moiety of GSK2251052 was oxidized to a propanoic acid moiety. Renal clearance of GSK2251052 predominated in rats, while metabolic clearance was significant in monkeys.

GSK2251052 is currently being evaluated in phase 2b studies for the treatment of complicated urinary tract and intra-abdominal infection [88].

Benzoxaboroles represent an exciting novel class of anti-infectives that inhibit a novel target. A derivative with a C6-substitution inhibited LeuRS from the *Trypanosoma brucei* parasite with $IC_{50} = 1.6\,\mu M$ has demonstrated parasite growth inhibition, potentially expanding the utility of these agents to parasites [89].

16.4 Considerations for the Development of aaRS Inhibitors

16.4.1 Resistance Development

Most aaRS inhibitors fit tightly in the binding pocket of the enzyme, thereby preventing the natural amino acid substrate from binding and being activated for condensation with the tRNA. Given the high selective pressure in the presence of antibacterial agents and the fact that aaRS inhibitors typically exert a bacteriostatic effect, cells acquire specific point mutations in the target gene that reduces the affinity of the drug, but still allows the natural substrate to bind. Such spontaneous target mutations have been extensively studied for IleRS in *S. aureus* exposed to mupirocin, where most first-step mutants carried V588F or V631F IleRS variants, and second-step mutants with even higher drug tolerance had additional mutations [90]. Similarly, REP8839, a MetRS inhibitor, induced I57N and G32S substitutions around the active site of *S. aureus* MetRS, and such mutants were less susceptible to this agent [91]. Indolmycin resistance occurs as a consequence of the TrpRS target mutation H43N, a residue directly involved in the binding of tryptophan and in the stabilization of the tryptophanyl-adenylate intermediate [36]. Resistance frequencies with which aaRS target mutations arise are in the range of 10^{-8} to 10^{-9}, and mutations are often associated with a fitness burden seen as a reduced growth rate due to a lower turnover of the synthetase. Other known modes of resistance to aaRS inhibitors include altered amino acid uptake or transport, overexpression of the synthetase, and induction or acquisition of a second synthetase that is not affected by the antibacterial agent [92]. To minimize the emergence of resistance when developing novel antibiotics that target aaRSs, one strategy would be to use such agents in combinations and/or at concentrations above the mutant prevention concentration [13]. Attractive are topical agents for wound infections or formulations to treat enteric infections where the drug is expected to accumulate to high levels at the site of infection without appreciable systemic exposure in order to maximize efficacy and minimize emergence of resistance.

16.4.2
Selectivity over Eukaryotic and Mitochondrial Counterparts

Selectivity of drug candidates for the bacterial enzyme compared to their human orthologs needs to be monitored to minimize potential toxicological effects. Mitochondrial aaRSs have a closer phylogenetic relationship to bacterial aaRSs compared to their cytosolic counterparts. Nonetheless, the eukaryotic aaRSs exhibit sufficient differences to enable development of highly selective inhibitors as evidenced by >1000-fold selectivity of MetRS [79, 93] and PheRS inhibitors [63] for the bacterial *versus* mitochondrial forms.

16.4.3
Spectrum of Activity

The immense diversity of aaRSs, as seen in their classification, and the structural differences between synthetases from different microorganisms, affect the spectrum of activity of old and new aaRS inhibitors. MetRS, for example, is found as a type 1 enzyme in most gram-positive bacteria, including *S. aureus*, but a second, type 2 MetRS that is more closely related to archeabacterial and gram-negative synthetases, is found in addition to the type 1 MetRS in a significant proportion of *S. pneumonia* strains [94]. Such preexisting natural resistance due to horizontal transfer of a gene encoding an alternate aaRS can seriously limit the usefulness of a novel class of antibacterial agents. In the case of the *S. pneumoniae* strains carrying both enzymes, this seriously hampered the prospect of this compound for respiratory tract infections. On the other hand, a limited spectrum of activity can be a desired advantage for targeted narrow-spectrum antimicrobial therapy. An example is CRS3123, a novel agent in clinical development for CDI that is inactive against most microbes of the normal gut flora and thus may provide a more selective treatment option compared to existing regimens [80].

16.4.4
Amino Acid Antagonism

The activity of aaRS inhibitors can be compromised by substrate antagonism, in particular if the inhibitor is a close aaRS substrate analog that utilizes the same uptake and transport systems. A recent example of this phenomenon is the phenyl-thiazolyl-sulfonamide PheRS inhibitor that possesses potent activity against major respiratory pathogens, including *S. aureus* (MIC $< 1\,\mu g\,ml^{-1}$), but was inactive in the presence of 100 μM Phe [63]. Poor *in vivo* efficacy was observed in a *S. aureus* sepsis model in mice with normal Phe blood levels; however, the compound was efficacious in mice that were fed a Phe-free diet and had low Phe blood levels.

16.5 Conclusions

In conclusion, aaRSs are validated drug targets, as many known natural compounds inhibit their function. They remain attractive targets for screening of compound libraries, although such efforts over the past 20 years have shown mixed success. Typically, aaRSs for amino acids with large or medium side chains are more likely to yield biochemical inhibitors, as the binding pockets for the larger substrates can accommodate such compounds, while inhibitors of aaRSs that catalyze the condensation of amino acids with small side chains such as Gly or Ala have not been found.

There are many lessons and pitfalls learned from using aaRSs in screening for novel antibiotics. Many compounds that inhibited a biochemical synthetase assay failed to exhibit antibacterial activity for a variety of reasons discussed earlier. Some were not useful hits as they possessed an antimicrobial mode of action that was not due to specific aaRS inhibition, and many compounds showed an unfavorable physicochemical or pharmacological profile. Further considerations for developing an aaRS inhibitor include resistance development, spectrum of activity, selectivity, and substrate antagonism.

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17
Antibiotics Targeting Translation Initiation in Prokaryotes

Cynthia L. Pon, Attilio Fabbretti, Letizia Brandi, and Claudio O. Gualerzi

17.1
Introduction

Approximately half of all known antibiotics target the translational apparatus [1–4], but because very few of them are specific inhibitors of the initiation phase of protein synthesis, translation initiation can be regarded as being a particularly underexploited antibiotic target.

Furthermore, as initiation is the phase of protein synthesis displaying the greatest evolutionary divergence among all translation steps, the kingdom-specific characteristics of the initiation mechanisms render prokaryotic translation initiation a potentially unique and selective target of inhibitors directed against bacteria. This translation phase is also a potential antibiotic target within prokaryotic-type organelles (apicoplasts and mitochondria) present in protozoan parasites such as *Plasmodium* sp. and *Toxoplasma* sp. [5, 6].

These circumstances qualify translation initiation as an ideal target for the urgently needed new anti-infectives having novel modes of action and possibly novel chemical structures for which resistance mechanisms have not yet been developed in nature [1–3].

For a better reference to the subject of this chapter and for a better understanding of the mechanism of action of translation initiation inhibitors, we present subsequently a short description of translation initiation in bacteria. Furthermore, a mechanistic model compatible with all available experimental data of the events occurring immediately before and during formation of 30S initiation complex (IC) and 70S IC is schematically presented (Figure 17.1 and Figure 17.2). The specific steps targeted by the individual inhibitors are also indicated.

17.2
Mechanism of Translation Initiation

Translation initiation is a multistep process in which both efficiency and fidelity are subject mainly to kinetic controls. In prokaryotes, protein synthesis begins

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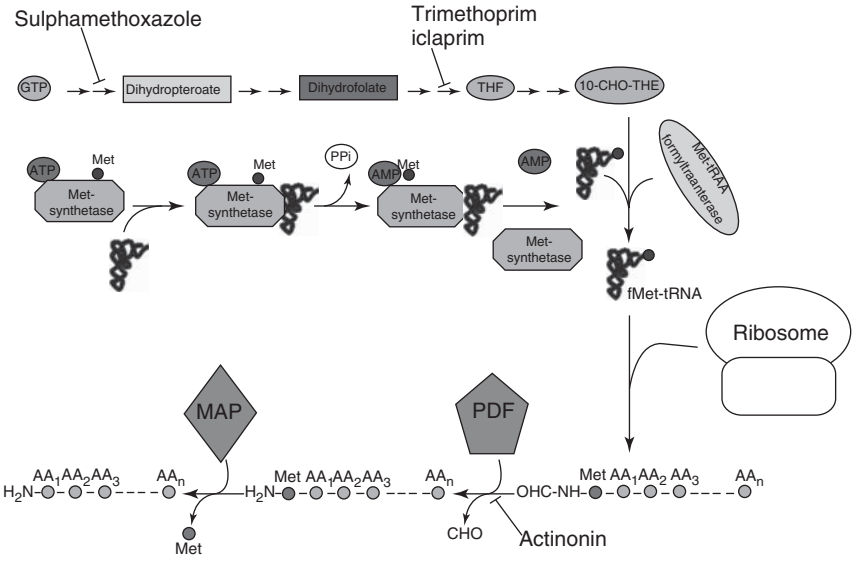


Figure 17.1 The folate metabolism, aminoacylation, and formylation of initiator Met-$tRNA_{fMet}$ and product deformylation. Schematic representation of the steps involved in the formation of fMet-$tRNA_{fMet}$ and in the deformylation of the nascent polypeptide, with an indication of the individual steps inhibited by the antibiotics mentioned in the text.

with $tRNA_{fMet}$, a special type of tRNA that is significantly different from elongator tRNAs from both structural and functional points of view, being designed to bind preferentially to the P-site instead of the A-site. The $tRNA_{fMet}$ is charged with methionine by the same Met-tRNA synthetase that charges elongator $tRNA_{Met}$ and is subsequently recognized by a formylase which transfers a formyl group from tetrahydrofolate to the α-NH_2 of Met (Figure 17.1).

Because inhibition of these processes will automatically interfere with translation initiation, not only in bacteria but also in the apicoplasts of the apicomplexan parasites, antibiotics interfering with these activities are briefly described in subsequent text. In light of the fact that inhibition of the Met-tRNA synthetase is extensively treated by Ochsner and Jarvis in Chapter 16, we shall restrict our discussion to the inhibitors of the formylation and deformylation processes.

The early event in translation initiation consists in the assembly of a 30S pre-IC that contains the three initiation factors IF1, IF2-GTP and IF3, the mRNA, and fMet-tRNA, the latter two ligands not having established a stable interaction with each other [7–9]. The 30S pre-IC undergoes a first-order isomerization, which is rate limiting, to form a more stable, or ''locked'' 30S IC [7–9] (Figure 17.2). This isomerization entails the decoding of the mRNA initiation triplet by the anticodon of the fMet-tRNA in the P-site of the 30S subunit. This process is accompanied by an adjustment on the subunit surface of the positions of IF3 and likely also of IF1 and IF2. If initiation triplet, mRNA structure, and tRNA are ''canonical,'' a correct complex is formed, whereby the affinity of IF3 for the subunit is reduced in

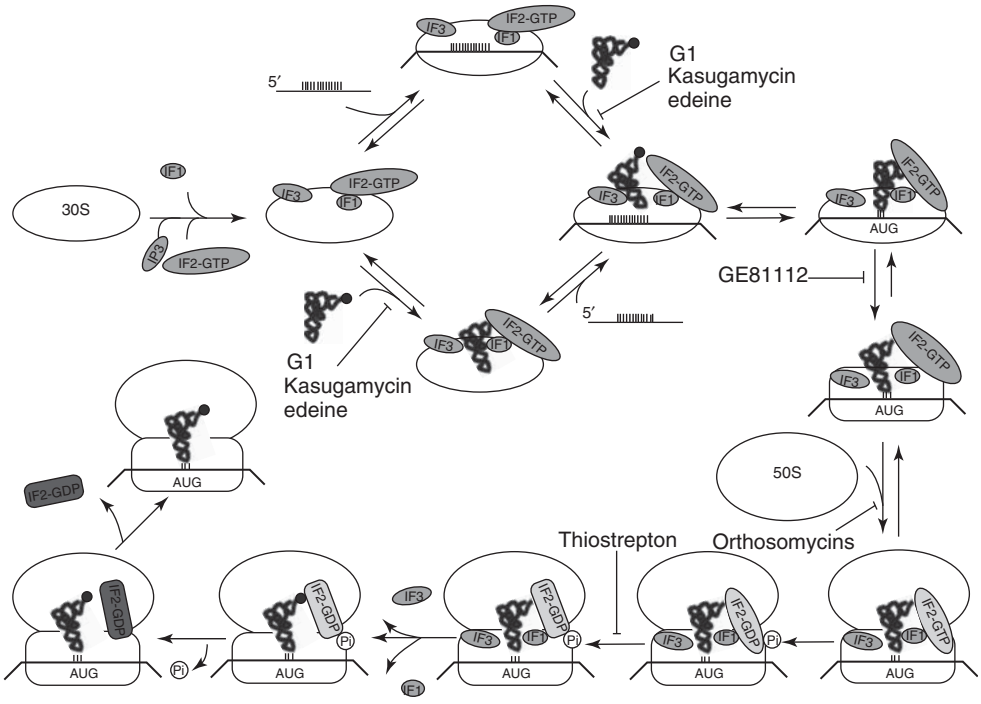


Figure 17.2 Mechanism of translation initiation in prokaryotes Schematic representation of the early and late events occurring during initiation of bacterial protein synthesis as described in the text. The individual translational steps inhibited by the antibiotics described in the text are also indicated.

preparation for its dissociation that will take place during the subsequent docking of the 50S subunit with the 30S IC [10, 11]; furthermore, under the influence of IF1 the 16S rRNA will assume an ''initiation-favorable'' conformation around A1408 in helix h44 and the 30S IC will acquire an overall best-fit conformation [10, 11] which will allow its fast docking by the 50S subunit [10, 12]. However, if the codon is not one of the ''canonical'' initiation triplets (i.e., AUG, UUG, and GUG), if the mRNA possesses an awkward structure (e.g., has a too long SD sequence), or the tRNA is not the standard initiator fMet-tRNA, IF3 will remain more stably bound and will favor the dissociation of the ''noncanonical'' 30S IC [13–16]. This most likely occurs through an effect of IF3 on the conformational dynamics [17] of the molecular gate separating the P- from the E-site; this gate is constituted by 16S rRNA bases G1338, A1339 on one side, and A790 on the other [18–21]. IF1 will also contribute to the fidelity function of IF3 by conferring an unfavorable conformation upon helix h44 [10, 11]. Overall, kinetic control of the correct formation of the 30S IC by the IFs represents the first checkpoint for translation initiation fidelity.

The preferential order in which the 30S subunit binds its ligands during the assembly of the 30S pre-IC has been determined by fast kinetics analysis [22]. IF3

and IF2 are the first factors to arrive, forming an unstable 30S–IF2–IF3 complex. Then IF1 joins in, locking the factors in a more stable complex. The correctly aminoacylated and formylated initiator fMet-tRNA is then recruited to the 30S subunit by 30S-bound IF2 which, unlike thermo-unstable elongation factor (EF-Tu), does not function as a tRNA carrier [9, 23]. The initial binding of mRNAs and their affinity for the 30S subunit are independent of initiation factors [22, 24–26] and of fMet-tRNA, and can take place at any time during 30S pre-IC assembly. These findings confirm the early demonstration that fMet-tRNA and mRNA bind in stochastic order to the small subunit [7] and that the IFs do not affect, not even very marginally, the thermodynamic affinity of the ribosome for these ligands [24, 25].

The transition from 30S IC to 70S IC (Figure 17.2) has also been studied by kinetic analysis [10, 27–29]. The main conclusions drawn are (i) 30S IC docking by the 50S subunit is accompanied by the ejection of IF3 followed by IF1 with the rate of dissociation of these factors depending on the nature of the 30S IC (i.e., canonical, noncanonical, non-best-fit), thereby establishing a second level of kinetic selection of the ''correct'' complex; (ii) upon 30S IC-50S association, IF2-bound GTP is immediately hydrolyzed, while the γ-Pi dissociation from ribosome-bound IF2-GDP-Pi occurs later and is rate limiting for accommodating fMet-tRNA in the P-site to yield a productive 70S IC; (iii) the 70S IC is formed in different steps that entail successive conformational changes of both fMet-tRNA and IF2; and (iv) the free energy generated by GTP hydrolysis is not necessary for the process [30], but failure to dissociate the γ-Pi from IF2 (as with the nonhydrolyzable analogs GDPNP or GDPCP) leaves the factor stuck in a conformation that does not allow the dissociation of its C2 domain from the acceptor end of fMet-tRNA and prevents IF2 from leaving the ribosomes and fMet-tRNA from acting as a donor in initiation dipeptide formation (for reviews on the subject, see [31–33]).

17.3 Inhibitors of Folate Metabolism

Antibiotics such as sulfametoxazole (Figure 17.3, structure **1**) and the dihydrofolate reductase (DHFR) inhibitors trimethoprim and iclaprim (Figure 17.3, structures **2** and **3**) are well known and have been successfully used in therapy. Other inhibitors of folate metabolism such as proguanil, pyrimethamine, and dapsone (Figure 17.3, structures **4–6**) have also been investigated for the possible treatment of infections by protozoan parasites such as *Plasmodium falciparum* [34]. All these molecules can be considered translation initiation inhibitors insofar as they interfere with the metabolic chain leading to the formation of initiator fMet-tRNA$_{fMet}$ from Met-tRNA$_{fMet}$ (Figure 17.1). However, none of these nor similar molecules can be regarded as exclusive translation initiation inhibitors as they inhibit also other cellular functions, such as DNA replication, requiring thymidine, whose biosynthesis depends on tetrahydrofolic acid as a precursor.

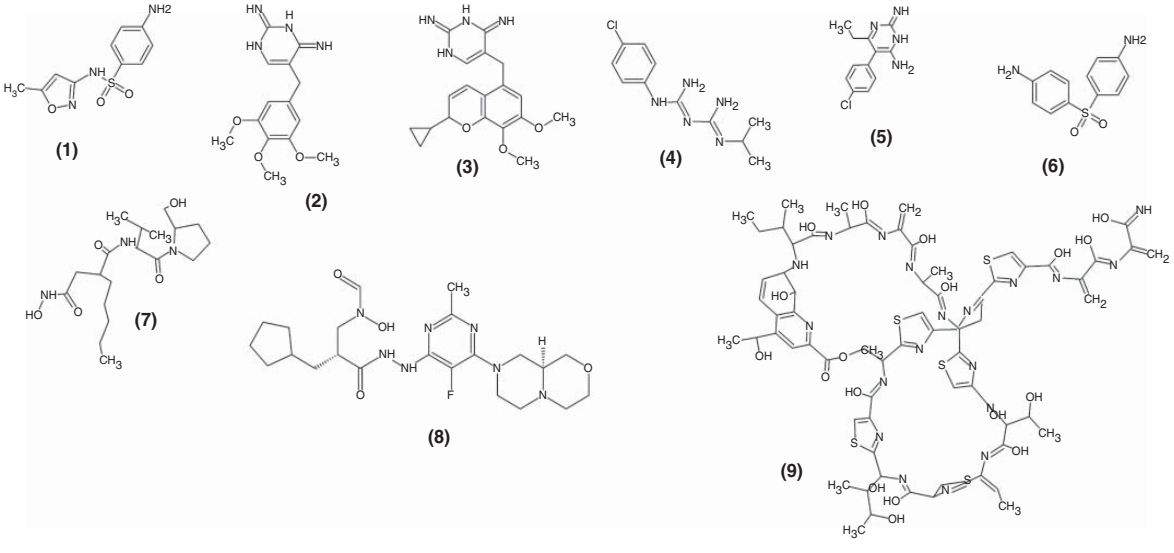


Figure 17.3 Chemical structures of the antibiotics described in this chapter sulfametoxazole **1**, trimethoprim **2**, iclaprim **3**, proguanil **4**, pyrimethamine **5**, dapsone **6**, actinonin **7**, GSK1322322 **8**, thiostrepton **9**, evernimicin **10**, avilamycin **11**, ppGpp analog **12**, kasugamycin **13**, edeine **14**, pactamycin **15**, GE81112 **16**, and Furvina® **17**. (*Continued overleaf*)

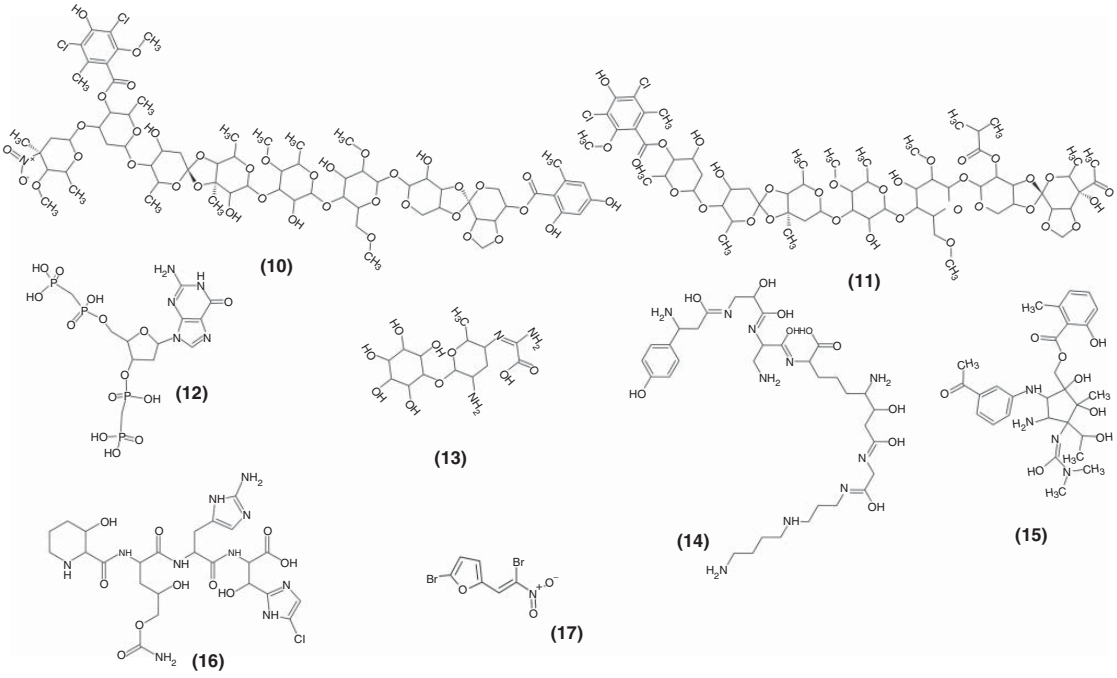


Figure 17.3 (*Continued*)

17.4
Methionyl-tRNA Formyltransferase

A transferase that adds the formyl group to the α-NH_2 group of Met-$tRNA_{fMet}$ (Figure 17.1) is an exclusive characteristic of the bacterial kingdom [35] and is also present in the apicoplasts of apicomplexa protist parasites such as *Toxoplasma gondii* and *Plasmodium* sp., where this enzyme is nuclearly encoded and targeted to the organelle by a specific signal peptide [36]. In light of its kingdom specificity, of the severe phenotype displayed by bacteria incapable of formylating their initiator tRNA [37], and of the availability of its crystal structure [38], methionyl-tRNA formyltransferase seems to have many of the characteristics of an excellent target for antibacterial drugs. However, no effective inhibitor of this enzyme has been found so far and therefore this enzyme remains one of the several unexploited antibiotic targets within the translational apparatus.

17.5
Inhibitors of Peptide Deformylase

As a consequence of the formylation of initiator Met-tRNA, which occurs before its decoding in the ribosomal P-site, the nascent peptides synthesized in prokaryotes, unlike those in eukaryotes, have a formy-methionine at their N-terminus. However, during maturation of the polypeptide, the *N*-formyl group must be removed from the newly synthesized polypeptide, after which the N-terminal methionine is also often removed. The hydrolytic removal of the formyl group (Figure 17.1) is carried out by a metal (Fe^{2+} in most cases) enzyme peptidyl deformylase (PDF) [39, 40] and the N-terminal methionine is removed by methionine aminopeptidase (MAP) from some but not all newly synthesized proteins [41]. The discovery that PDF, a metalloprotease, is conserved, ubiquitous, and essential in bacteria [42] but not present in eukaryotic cells (aside from the mitochondria and plastids) [43], prompted a rush to find inhibitors of this molecule, considered to be a potential target for novel antibacterial agents.

The first reported PDF inhibitor, actinonin (Figure 17.3 structure **7**), was found by screening a chemical library of compounds containing metal ion chelating groups. Actinonin is a naturally occurring antibiotic produced by *Streptomyces* species with a hydroxamate group that acts as the chelating group to bind the metal ion of the enzyme and a tripeptide binding domain. Although it had been discovered to be an antimicrobial agent as early as 1962 [44], its target was not identified until 2000 when actinonin was found to be a tight-binding inhibitor of purified PDFs from *Escherichia coli* and *Staphylococcus aureus* and its antibacterial activity was correlated with its inhibition of deformylase activity [45]. Although the compound is able to penetrate the bacterial cell wall and membrane and enter the cell, it is rapidly exported in organisms with an efficient efflux pump so that an efficacious intracellular concentration of the drug could not be attained and *in vivo* actinonin is only moderately active against gram-positive bacteria and fastidious

gram-negative bacteria. Therefore, actinonin was not further developed. However, the discovery that actinonin is a potent PDF inhibitor *in vitro* prompted the search for and development of better PDF inhibitors by a combination of rational design based on multiple PDF inhibitor scaffolds and medicinal chemistry strategies. These approaches led to the development of a plethora of second-generation actinonin derivatives. The design, synthesis, and properties of many of these (pseudopeptidic hydroxamic acids or *N*-formyl-*N*-hydroxylamines) PDF inhibitors have been described in many reviews [46–49].

Two promising inhibitors obtained in these attempts to produce actinonin derivatives had advanced to phase I clinical trials, BB-83698 (British Biotech/Vernalis/Oscient) in 2002 and LBM-415 (Vicuron/Novartix) in 2003. Although both compounds seemed to have passed these trials without problems, the further development of both products was terminated for unspecified reasons, some of which possibly related to their weak potentiality as antimicrobials. In addition to the above-mentioned difficulty to maintain an effective concentration of the drug within cells, bacteria can easily develop resistance to PDF inhibitors and bypass the need for an active PDF by multiple pathways such as mutations in the formyl transferase gene (*fmt*) or in the methenyltetrahydrofolate dehydrogenase (*folD*) gene [48, 49].

However, Goemoaere *et al.* [50] have recently found that the use of a combination of different inhibitors acting on two tightly associated metabolic enzymes such as PDF and MAP resulted in a cooperative inhibitory effect that could be sufficient to combat multidrug-resistant bacteria.

Making use of a structure-based rational design strategy involving PDF cocrystallization studies and evaluation of PDF inhibition and antibacterial activity followed by screening of various PDF inhibitor scaffolds, GlaxoSmithKline has identified hydrazinopyrimidines as a new series of PDF inhibitors [51]. From this series, GSK1322322 (Figure 17.3 structure **8**) has emerged as a promising drug, which has recently successfully completed a phase II trial for acute bacterial skin and skin structure infections [52]. Furthermore, this molecule has shown potent activity against methicillin-resistant *Staphylococcus aureus* (MRSA), in addition to being active against respiratory pathogens *Haemophilus influenzae* and *Streptococcus pneumonia* [53].

17.6 Inhibitors of Translation Initiation Factor IF2

Thiostrepton (Figure 17.3 structure **9**) is a cyclic thiazolyloligopeptide antibiotic (MW 1664.83) produced by several streptomyces strains (e.g., *Streptomyces laurentii*) and arises from several posttranslational modifications of a ribosome-synthesized thiostrepton pre-peptide (TsrA) [54]; however, this antibiotic can also be obtained by total chemical synthesis [55, 56]. Thiostrepton is mainly used in veterinary medicine, in combination with other antibiotics, such as neomycin and nystatin, for the topical treatment of mastitis and of dermatological infections caused by

gram-negative pathogens; furthermore, thiostrepton was also shown to repress the expression of transcription factor forkhead box M1 (FOXM1), thereby exhibiting activity against breast cancer cells [57]. Despite these properties, its hydrophobic character and poor bioavailability have so far prevented its therapeutic use in humans. Nonetheless, other new synthetic thiopeptides effective against gram-positive bacteria, including MRSA, and against the *P. falciparum* apicoplast [58] may turn out to be more successful [59, 60].

Although thiostrepton is a well-documented elongation inhibitor that interferes with both EF-G-dependent and spontaneous translocation [61, 62], the possibility that thiostrepton might inhibit translation initiation was also considered, but this hypothesis was rejected when early studies yielded inconsistent results. Nevertheless, this issue was reopened by the finding that 23S rRNA cleavage by hydroxyl radicals generated by Fe(II)EDTA tethered to IF2 occurred in regions believed to represent the thiostrepton binding site (e.g., H43 and H44), while thiostrepton abolished the effects of IF2 on the exposure of the same 50S sites [63]. Thus, the possibility that this antibiotic might influence the IF2-50S interaction and/or initiation functions was reexamined and it was demonstrated that, in addition to its canonical translocation target, thiostrepton interferes with the ribosomal binding of IF2 and inhibits IF2-dependent initiation dipeptide formation. As dipeptide formation is reduced, ~80% at thiostrepton concentrations (1–2 mM) comparable to those required to inhibit EF-G activity to approximately the same extent, it was concluded that thiostrepton is equally effective in inhibiting initiation and elongation [63]. This inhibition was attributed to an interference with the interaction between IF2 and the ''L11 domain'' of the 50S subunit, which undermines the stability of the IF2-ribosome complexes and results in a nonproductive positioning of fMet-tRNA in the ribosomal P-site [63]. However, as fMet-puromycin, unlike initiation dipeptide formation, is not inhibited by thiostrepton and because transpeptidation requires the correct positioning of both the donor and acceptor substrates, the possibility that inhibition of aminoacyl-tRNA binding to the A-site by this antibiotic may contribute to the inhibition was investigated. In fact, although thiostrepton was reported to have only a negligible effect on ''initiation'' ribosomes (i.e., having both E-site and A-site empty and fMet-tRNA in the P-site), in contrast to the severe inhibition of the A-site function observed with ''elongation'' ribosomes, which bear a peptidyl-tRNA in the P-site and a deacylated tRNA in the E-site [62], more recent data showed that the EF-Tu-dependent binding of Phe-tRNA can be reduced (15–40% depending on the experimental conditions) by this antibiotic [63].

Subsequently, fast kinetic analyses demonstrated that the main effect of thiostrepton is to slow down 25-fold the first-order conformational transition that transforms the 70S·IF2·GDP·Pi·fMet-tRNA$_{fMet}$ complex into the 70S·IF2*·GDP·Pi·fMet-tRNA$_{fMet}$, an intermediate complex along the pathway that allows a conformational change or a repositioning of the initiator tRNA and the release of Pi (Figure 17.2) [28].

The ribosomal localization of thiostrepton, as surmised from the above-mentioned chemical probing experiments, was later confirmed and more precisely pinned down by the crystallographic study of the *Deinococcus radiodurans*

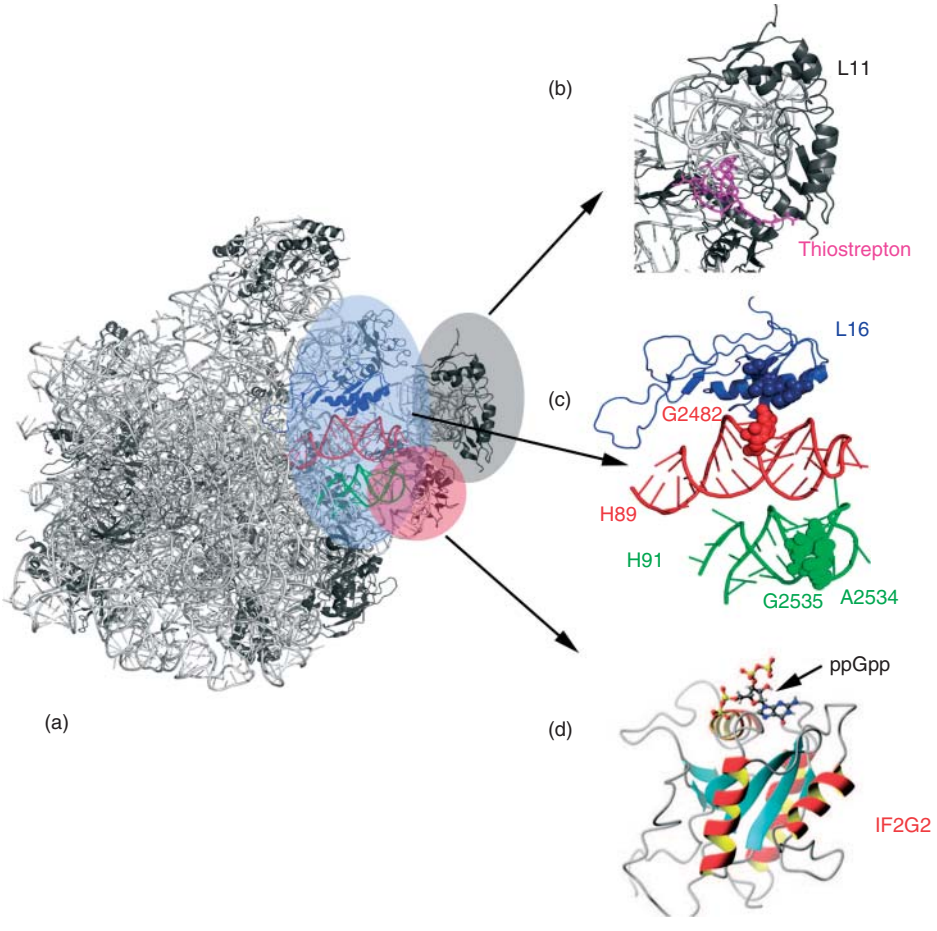


Figure 17.4 Inhibitors of IF2 function. (a) Structure of the 23S and 5S rRNA (light gray) and ribosomal proteins (black) in the 50S ribosomal subunit. The region of the subunit interacting with thiostrepton is highlighted in gray and expanded in (b); the region interacting with orthosomycins is highlighted in cyan and expanded in (c); the region interacting with the IF2-G2·ppGpp complex is highlighted in pink (d). Thiostrepton (magenta); ribosomal protein L16 (blue); 23S rRNA helices H91 (green), and H89 (red).

50S-thiostrepton complex (Figure 17.4a). The structure shows the antibiotic being located in the GTPase-associated center of the subunit, in a cleft formed by the N-terminal domain of ribosomal protein L11 and H43/H44 of the 23S rRNA (Figure 17.4b) [64].

Orthosomycins are a class of oligosaccharide antibiotics produced by various organisms; they are endowed with excellent antimicrobial activity against gram-positive bacteria, including multiply resistant strains of MRSA and vancomycin-resistant enterococci (VRE) as well as against some gram-negative bacteria [65].

This group of molecules comprises evernimicin and avilamycin (Figure 17.3 structures **10** and **11**), two inhibitors of bacterial ($IC_{50} = 125$ nM in cell-free extracts from either *E. coli* or *S. aureus*) and archaeal translation, but essentially inactive on translation with extracts of wheat germ and rabbit reticulocytes. Avilamycin is approved for use in veterinary medicine, as preventative for swine dysentery, and growth promotant in pigs. Evernimicin (Ziracin® by Schering-Plough) is a generally safe and well-tolerated antibiotic, still under development for serious infections caused by glycopeptide-resistant enterococci (VRE), staphylococci (*Staphylococcus epidermidis, aka* GISE, and *S. aureus* (GISA)), and *Streptococcus pneumoniae*. Evernimicin binds to *E. coli* and *S. aureus* ribosomes and 50S subunits with fairly high affinity (K_{diss} from 84 to 160 nM). As evernimicin binds to the ribosomes in competition with avilamycin, cross-resistance between these two antibiotics has been observed. Chemical probing of the rRNA and analyses of nucleotide substitutions giving rise to resistance indicate that binding of evernimicin involves a number of bases belonging to helices H89 and H91 of 23S rRNA. Indeed, evernimicin and avilamycin protect from chemical modification A2482 in H89 and A2534 in H91 [66, 67], while mutations in helices 89 and 91 confer resistance to evernimicin and a G2535A substitution causes avilamycin resistance [67, 68]. Thus, taken together these data indicate that the orthosomycins bind to a single high-affinity site at the base of the 50S stalk, close to the elbow of A-site tRNA, between the minor groove of H89 and the loop region of H91 (Figure 17.4c). As this site is not the target of other antibiotics, no relevant cases of cross-resistance with other drugs have been reported. This topographical localization of evernimicin corresponds to the region of 23S rRNA (i.e., A1476 and A2478 in H89) protected by IF2 from chemical probing [69] and ''covered'' by IF2 as seen in cryo-EM images [70, 71]. Thus, the orthosomycins likely interfere with the interaction of IF2 with the 50S ribosomal subunit, thereby preventing the association of the 30S IC with the 50S subunit to yield a 70S IC (Figure 17.2) and inhibiting IF2-dependent formation of fMet-puromycin (Figure 17.2) [66]. On the other hand, in agreement with the finding that evernimicin binds to the 50S ribosomal subunit, no interference of this antibiotic with IF2-dependent formation of 30S IC was observed. However, in addition to inhibiting protein synthesis, evernimicin was found to inhibit also the assembly of the 50S subunit [72]. The effects of thiostrepton and micrococcin on translational GTPases have been compared to those of evernimicin and it was demonstrated that while the two thiopeptides inhibit translocation, the orthosomycin antibiotic, in addition to inhibiting IF2-dependent 70S IC formation, severely inhibits EF4-mediated back-translocation [73]. However, it should be remarked in this connection that *lepA*, the EF4-encoding gene, is not essential in *E. coli*, so that it is unlikely that this factor plays an essential role that would justify the antibacterial activity of this antibiotic [74].

In addition to the aforementioned mutations of 23S rRNA, resistance to evernimicin in *S. pneumoniae* can result from G2470 methylation in H89 [75] and probably from an indirect perturbation of 23S rRNA resulting from single amino acid substitutions in ribosomal protein L16 (i.e., R56H, I52T, and R51H, Figure 17.4c) [68, 76].

17.7
ppGpp Analogs as Potential Translation Initiation Inhibitors

Upon nutritional stress (i.e., starvation of carbon, phosphorus, nitrogen, iron, or lack of an essential amino acid), the bacterial cell responds with what is known as the *stringent response*, which entails the rapid accumulation of a global regulator, the alarmone ppGpp. Two ribosome-associated proteins, RelA and SpoT, are responsible for the production of (p)ppGpp, starting from ATP and GDP or GTP [77 and references therein]; in turn, this enables the cells to survive nutrient deprivation [78] and causes a series of responses [79] among which are – for what is pertinent to the subject of this chapter—becoming resistant to antibiotics [80, 81], forming biofilms, producing antibiotics, and expressing virulence [82 and references therein].

Thus, in light of the aforementioned, in an attempt to develop novel antimicrobial agents directed against targets completely absent in mammalian cells, ppGpp analogs have been recently synthesized and shown to inhibit Rel proteins in both gram-positive and gram-negative bacteria by competing with ppGpp for binding to this bacteria-specific enzyme. In particular, the ppGpp analog indicated as ''compound 10'' (Figure 17.3, structure **12**) displays interesting properties so that it could represent a starting point for the preparation of novel antibacterial inhibitors [83]. Indeed, the use of ppGpp analogs may turn out to be useful also to render bacterial cells within biofilms more sensitive to antibiotic treatment. In fact, at least in some bacteria, the stringent response plays a role in rendering the cells antibiotic insensitive within biofilms. As it has been reported that the number of ofloxacin-susceptible cells in *Pseudomonas aeruginosa* biofilms is increased by three log units upon disruption of both RelA and SpoT genes [81], it can be expected that ppGpp mimics may take the place of the natural alarmone and interfere with whatever function ppGpp may have in biofilm antibiotic resistance. However, because ofloxacin failed to sterilize the biofilm of SpoT/RelA mutants [81], the use of ppGpp analogs cannot be expected to eradicate completely biofilm infections but only to alleviate the problem and contribute to their elimination in combination with other treatments.

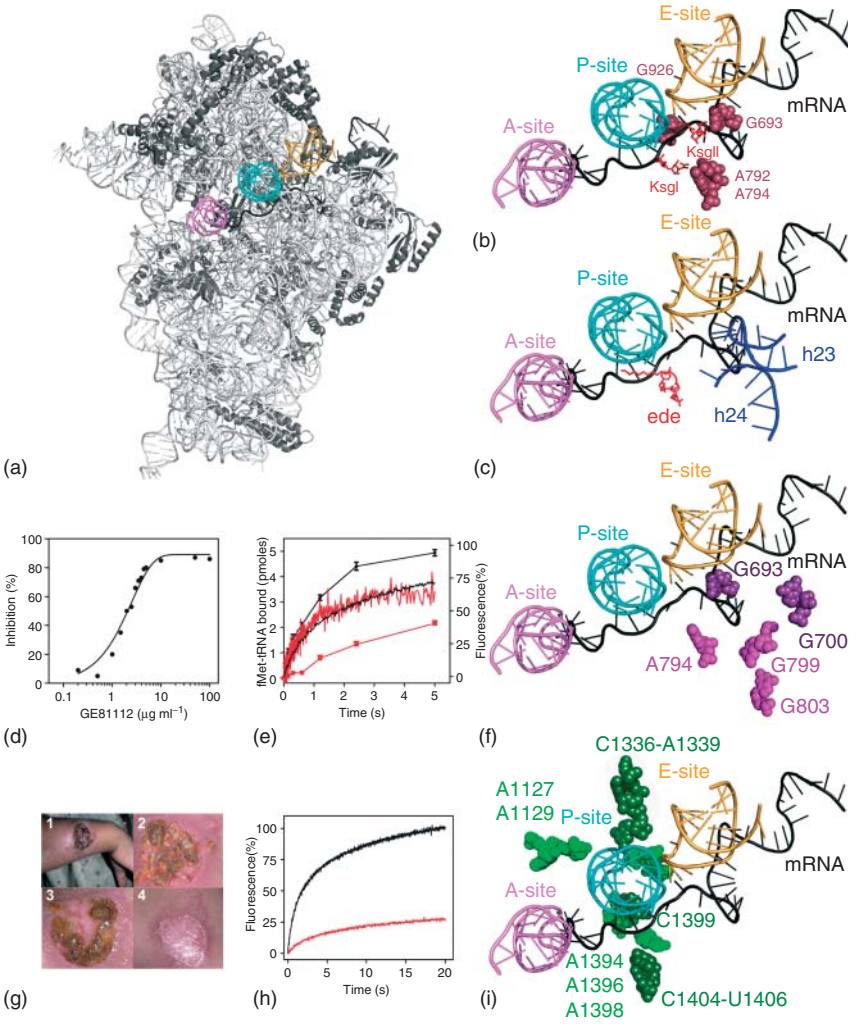
However, the additional possibility of using ppGpp analogs to inhibit translation initiation should also be considered. In fact, in addition to its well-known effect on transcription, whereby the function of the stringent-response-sensitive promoters of stable RNAs is inhibited [77], ppGpp is also capable of inhibiting translation [84, 85]. While ppGpp is known to bind to both elongation factors EF-Tu and EF-G, it has been shown that initiation factor IF2 represents a selective target of ppGpp inhibition [84] so that this alarmone can be considered a physiologically relevant IF2 ligand, which binds with similar affinity to the same nucleotide-binding site as GTP [84]. Thus, under nutritional stress, when the metabolic conditions of the cell do not allow a high level of protein synthesis, the intracellular concentration of ppGpp increases dramatically and IF2 can bind the alarmone in alternative to GTP, thereby inhibiting new translational events. Binding of ppGpp interferes with IF2-dependent fMet-tRNA binding, severely inhibits initiation dipeptide formation,

and blocks the IF2-dependent initiation step of mRNA translation. As GDP, unlike ppGpp, does not inhibit the IF2 function, it can be surmised that the inhibitory effects of the alarmone is most likely due to the presence of a diphosphate at the 3′ position of this molecule; by protruding from the G2 domain (Figure 17.4d), the negatively charged diphosphate likely interferes with other domains of IF2 and/or with its interaction with one or more of its ligands such as the ribosomal subunits and the fMet-tRNA [84].

17.8 Translation Initiation Inhibitors Targeting the P-Site

Regarding the inhibitors targeting the 30S P-site function within the initiation pathway, by far the most effective and ''initiation specific'' are GE81112, Furvina (or G1), and Kasugamycin. Other inhibitors (e.g., edeine and pactamycin) inhibit also other steps of translation or even other cell functions in addition to protein synthesis.

Kasugamycin (or Kasumin®) is a naturally occurring aminoglycoside (2-amino-2-[(2*R*,3*S*,5*S*,6*R*)-5-amino-2-methyl-6-[(2*R*,3*S*,5*S*,6*S*)-2,3,4,5,6-penta-hydroxy-cyclohexyl]oxyoxan-3-yl]iminoacetic acid) whose structure (Figure 17.3, structure **13**) was elucidated in the mid 1960s [86, 87]. Kasugamycin is produced by *Streptomyces kasugaensis* isolated in the Kasuga shrine in Nara [88], but can also be chemically synthesized [89]. Kasugamycin, which is a good agricultural biocide with low toxicity to humans, animals, and plants, was found to bind to the 30S subunit and 70S ribosomes with $K_{ass} = 6 \times 10^4$ M^{-1} and to inhibit translation initiation without affecting elongation [90, 91]. Early *in situ* chemical probing experiments carried out on drug–ribosome complexes demonstrated that kasugamycin protects the universally conserved A794 and G926 bases of 16S rRNA, which are also protected by P-site-bound tRNA; in addition, it causes an enhanced reactivity of C795 [92]. Subsequently, the X-ray structure of ribosome–kasugamycin complexes has been determined at 3.35 and 3.5 Å resolution with *Thermus thermophilus* 30S subunits and *E. coli* 70S ribosomes, respectively [93, 94]. Kasugamycin was found to bind between the head and the platform of the small subunit, within the mRNA channel at the top of h44 and between h24 (A794, A792) and h28 (G926) of 16S rRNA (Figure 17.5b). The binding site overlaps the kink between the P- and E-site codons of the mRNA (from position −2 to +1), (Ksg I, Figure 17.5b) [93, 94]. In addition, kasugamycin binds to a second site (Ksg II, Figure 17.5b), making contact with h23 (G693) and h24 as well as with ribosomal protein S7 in a position where it overlaps both mRNA and tRNA in the E-site [93, 94]. As kasugamycin is bound in the vicinity of but not directly in the P-site, its inhibition is likely due to an indirect perturbation of the geometry of the mRNA channel, which causes a distortion of the mRNA position and ultimately prevents correct codon–anticodon interaction. Thus, kasugamycin interferes with the correct P-site binding of fMet-$tRNA_{fMet}$ and induces the dissociation of P-site-bound tRNA [93–96].Unlike other aminoglycosides (e.g., streptomycin and kanamycin), which target the 30S A-site function and cause misreading (see Chapter 19 by Kondo and



Westhof in their book), kasugamycin does not cause miscoding, frame-shifting, or read-through of stop codons. Instead, it was actually found to enhance accuracy of protein synthesis [97]. Of particular interest is the fact that kasugamycin does not inhibit with the same efficiency the translation of all types of mRNA and therefore interferes with the expression of most but not all genes [98]. In general, the effects of kasugamycin exposure are pleiotropic [99] and the changes in protein expression pattern induced by this aminoglycoside are more severe than those observed in an *infA* mutant strain [100]. The different extents of inhibition by kasugamycin, observed in the translation of different templates, depends on how well the drug can compete with the individual mRNAs. As it turns out, translation of leaderless mRNAs, unlike that of leadered mRNAs, is insensitive to kasugamycin inhibition *in vivo* [101, 102] and is restricted to ribosomes that, likely as a consequence

Figure 17.5 Inhibitors of the 30S P-site function. (a) Structure of the 16S rRNA (light gray) and ribosomal proteins (dark gray) in the 30S ribosomal subunit in which the positions of A-site (magenta), P-site (cyan) and E-site (ocher) tRNAs as well as mRNA (black) are indicated. (b) Expanded view of the P-site decoding region showing the position of the 16S rRNA bases (bordeaux) involved in kasugamycin (red) binding; (c) expanded view of the P-site decoding region showing the position of the 16S rRNA helices h23 and h24 (blue) involved/influenced in/by edeine (green) binding; (d) inhibition of protein synthesis by increasing concentrations of GE81112 in a cell-free extract of a clinical isolate of *P. aeruginosa;* (e) kinetics of mRNA-dependent fMet-tRNA binding to the 30S in the absence (black tracings) or in the presence (red tracings) of GE81112. Binding was followed by fluorescence stopped-flow analysis (continuous lines) and by fast filtration using a quenched-flow apparatus (lines with experimental points); (f) expanded view of the P-site decoding region showing the position of the 16S rRNA bases protected from (purple) or exposed (magenta) to *in situ* modification by GE81112; (g) effect of Dermofural®, Furvina (G1) good cicatrization of leishmaniasis lesions observed because of elimination of opportunistic bacterial and fungal infections commonly found in this kind of disease. Images were taken during a preliminary clinical trial performed in Honduras in 2004 and obtained through courtesy of Drs Nilo R. Castanedo and Ricardo Medina (Centro de Bioactivos Quimicos, Universidad Central ''Marta Abreu'' de Las Villas, Santa Clara, Cuba). Further explanations can be found in the text and/or in the original literature quoted in the text. (h) Fluorescence stopped-flow kinetics of mRNA-dependent fMet-tRNA binding to the 30S in the absence (black tracings) or in the presence (red tracings) of Furvina (G1); and (i) expanded view of the P-site decoding region showing the position of the 16S rRNA bases protected (light green) or exposed (dark green) by Furvina (G1) to *in situ* cleavage by hydroxyl radicals. In (b,c,f,i), the positions of the mRNA and of the A-site, P-site, and E-site-bound tRNAs are indicated with the same colors used in (a).

of kasugamycin binding, have lost six (S1, S2, S6, S12, S18, S21) and possibly more ribosomal proteins [103]. These r-protein-deficient ribosomes can translate leaderless mRNAs in the presence of the drug while being translationally inactive vis-à-vis leadered mRNAs. Whether this is due to a failure of kasugamycin to bind stably to these ''incomplete'' particles or to another mechanism is not known.

Unlike with GE81112 (see subsequent text), inhibition by kasugamycin is not selective for bacterial translation initiation insofar as this antibiotic is also active in eukaryotic systems, where its mechanism of inhibition is likely similar, if not identical. In fact, kasugamycin binds also to eukaryotic 18S rRNA in a position likely equivalent to that which is targeted in bacteria [104]. The capacity of kasugamycin to inhibit fungal protein synthesis indeed justifies its original use in crop protection and, in particular, in the control of rice blast disease [105].

Furthermore, it has been suggested that kasugamycin inhibition might be targeted also against cellular processes other than translation. Indeed, one of the most common kasugamycin resistance mutations is that inactivating *ksgA* [106], the gene encoding KsgA which methylates nucleotides A1518 and A1519 involved in 16S rRNA maturation and in the establishment of the functional conformation of the 30S subunit during the final stages of ribosome assembly [107, 108]. Other mutations causing kasugamycin resistance were found to affect ribosomal proteins S2 [109] and S9 [110] (the latter giving rise to kasugamycin dependence) and G926 and A794 [111], the bases corresponding to the antibiotic binding site [93, 94].

In light of this, it seems surprising that these base mutations do not inhibit the ribosomal binding of the drug [93, 94].

Edeine (Figure 17.3, structure **14**) is a complex of four basic pentapeptides, but the antibiotic activity is only a property of A1 (N^a[(N^2-{N^2-[*N*-(*b*-tyrosyl)isoseryl]-2,3-diaminopropionyl}-2,6-diamino-7-hydroxyazela-9-yl)glycyl]-spermidine and of its analog B1. The drug, produced by *Bacillus brevis*, is microbiologically active against gram-positive and gram-negative bacteria as well as against some fungi.

Edeine interacts with universally conserved bases in h24, h28, h44, and h45 [92, 112] involved in translation initiation; it is therefore a ''universal'' inhibitor that blocks IC formation not only with prokaryotic but also with archaeal and eukaryotic ribosomes. In eukaryotes, edeine also prevents translation initiation from internal mRNA sites (IRES) and is active only on mRNA-coded ribosomes [113]. Edeine reduces the affinity of peptidyl-tRNA for the P-site of the 30S subunit as a result of an interaction of its spermidine moiety with the backbone of this ligand (Figure 17.5c), while the rest of the edeine molecule causes a distortion of h24 that induces an interaction between the loops of h24a and h23b sustained by cross-helix pairing between C795 and G693 [112]. This interaction imposes constraints on the mobility of the subunit in a region that is critical for its interaction with tRNA, mRNA, and IF3. This causes a perturbation of the mRNA channel, mainly in the initiation codon–anticodon and SD–anti-SD base-pairing regions. These effects and a direct clash of the antibiotic with initiator tRNA in the ribosomal P-site interfere with the binding of the initiator tRNA and cause the inhibition of 30S IC formation.

Furthermore, edeine was also found to induce an allosteric effect on the A-site decoding fidelity causing mRNA misreading, an effect not seen with other P-site inhibitors such as GE81112 and Furvina (see subsequent text). Finally, the effects of edeine, in addition to not being kingdom-specific, also lack target specificity because *in vivo* this antibiotic preferentially inhibits DNA synthesis [114, 115].

The tricyclic compound pactamycin (Figure 17.3, structure **15**) is a natural product of *Streptomyces pactum*, which was initially reported to inhibit IFs-dependent P-site binding of fMet-tRNA and was therefore originally classified as a translation initiation inhibitor [1]. However, later studies have demonstrated that this antibiotic does not cause any inhibition before the first translocation event. The inhibition is due to a blockage of 30S flexibility induced by pactamycin binding, which causes a distortion of the mRNA in the E-site thereby hindering the movement of the mRNA–tRNA complex from the A- to P- and from the P- to E-sites. As to the mechanism by which pactamycin induces its effects, it has been shown that this molecule binds to the 30S subunit and protects from chemical modification G693 and C795 [92], two bases also protected by P-site-bound tRNA; accordingly, the A694G, C795U, and C796U mutations confer pactamycin resistance [116]. As G693 and C795 are universally conserved, it is not surprising that this antibiotic is a potent translational inhibitor in all kingdoms of life. The structure of the 30S-pactamycin complex solved by crystallography [117] confirmed to a large extent the localization suggested by the chemical probing experiments, showing that the pactamycin-binding site overlaps both mRNA and tRNA in the E-site and coincides almost perfectly with the second kasugamycin-binding site (Figure 17.5b). Furthermore,

the crystallographic data demonstrating that the two lateral aromatic rings of pactamycin are stacked together on G693 at the tip of h23b, mimicking an RNA dinucleotide, while its central ring interacts with C795 and C796 in h24a and, to a lesser extent, with A694 (h23b). These effects are opposite to those caused by edeine which, as mentioned earlier, induces G693–C795 pairing; therefore, it is not surprising that pactamycin reverses the inhibition of edeine by breaking the G693–C795 base pair and restores the 30S conformation with a vacant P-site, allowing P-site binding of tRNA but not its translocation. The aromatic moieties of pactamycin occupy the position of the last two bases of the E-site codon. These interactions cause the locking of h23b with h24a and, as mentioned, determine a substantial distortion of the mRNA path that reduces the conformational dynamics of the 30S subunit. This distortion prevents mRNA movement through the 30S subunit and prevents the SD–anti-SD interaction.

GE81112 is a hydrophilic, chlorine-containing, noncyclic tetrapeptide constituted by nonproteinogenic L-amino acids. This antibiotic is endowed with a novel chemical structure, found in nature in three variants (A, B, and B1 with molecular masses of 643–658 Da) the most active of which proved to be the variant B (658 Da) (Figure 17.3, structure **16**) [118]. The synthesis of GE81112 is nonribosomal and 14 biosynthetic genes (*getA–N*) involved in its synthesis have been identified within a larger biosynthetic cluster cloned from *Streptomyces* sp. L-49973, which has been sequenced and partially characterized [119].

GE81112 is effective on almost all gram-positive and gram-negative bacteria tested, including clinical isolates of *P. aeruginosa* (Figure 17.5d), but has the drawback of being active only in minimal medium. In fact, in the absence of competition by other oligopeptides, GE81112 is actively pumped by the OPP (oligopeptide permease) system inside the cell where it is actually concentrated so as to account for the low MIC ($\leq 0.2\ \mu g\ ml^{-1}$) observed. However, in a rich medium the MIC is increased by approximately three orders of magnitude due to competition by other peptides for the OPP system, while the highly hydrophilic character of the molecule does not allow its spontaneous diffusion through the bacterial membranes [118, 120]. Nevertheless, research still in progress has shown that, upon targeted modifications introduced into GE81112 to increase its lipophilicity, the less hydrophilic molecules obtained can enter the cell bypassing the OPP pump, reaching MIC values that show potential for further improvement. The modified molecule maintains the antibacterial spectrum of the parent molecule and is directed against the same target (unpublished results).

GE81112 inhibits exclusively bacterial translation initiation and is the most specific inhibitor of this bacterial function found so far. This compound is also superior, at least *in vitro*, to other antibiotics traditionally classified as ''P-site inhibitors'' (edeine, pactamycin, and kasugamycin), as far as inhibitory efficiency and target selectivity are concerned [121]. GE81112 inhibits fMet-tRNA binding, and fast kinetics analyses demonstrate that the inhibition does not occur during the formation of the 30S pre-IC but instead concerns the subsequent step, namely, the first-order isomerization of the 30S pre-IC that yields the *bona fide* 30S IC (Figure 17.2 and Figure 17.5e)

Chemical probing [121] and protection from hydroxyl radical cleavage (unpublished results) show that some of the bases whose exposure is affected by GE81112 are close to or partially overlapping those implicated by other P-site inhibitors (Figure 17.5f). Recent crystallographic data on the 30S–GE81112 complex fully confirm the localization of the inhibitor in the P-site (unpublished results). Furthermore, these experiments indicate that the binding of this antibiotic causes also a generalized perturbation of the 16S rRNA structure around the P-site, which involves mainly helices h33 and h44. This GE81112-induced conformational change causes both fMet-tRNA and mRNA to assume a faulty position on the subunit surface, which prevents them from forming the canonical codon–anticodon base-pairing characteristic of a locked 30S IC. Instead, the 30S complex formed in the presence of GE81112 has an altered structure, unfit for optimal docking by the 50S subunit. Therefore, the transition 30S IC → 70S IC and the dissociation of the initiation factors IF1, IF2, and IF3, which accompanies this process, are slowed down in the presence of this antibiotic.

In conclusion, GE81112 belongs to a structurally novel class of molecules and inhibits efficiently and selectively an underexploited target within the translational apparatus. Thus, GE81112 represents a promising scaffold for designing new antibiotics and/or a substrate molecule for chemical modifications that would allow the drug to enter the cells bypassing the OPP pump so as to obtain effective, broad-spectrum antimicrobial agents for which resistance mechanisms are unlikely to exist.

Furvina®, also known as *G1 (MW 297)*, is an antibiotic developed in Cuba from sugarcane bagasse. It is a nitrovinylfuran [2-bromo-5-(2-bromo-2-nitrovinyl)-furan] (Figure 17.3, structure **17**) microbiologically active, not only against bacteria but also against pathogenic yeasts and filamentous fungi [122 and references therein], and therefore suitable for topical treatment of a wide range of human cutaneous infections (Figure 17.5g). Indeed, Dermofural®, an ointment containing G1 as the only active principle, was registered in Cuba in 2007 (Registro del Dermofural 0.15%. No. M-07-020-D01, CECMED, Centro para el Control Estatal de la Calidad de los Medicamentos. Ciudad de la Habana, 2007) and has since been used nationwide for the therapy of dermatological infections without causing any relevant side effect. In consideration of its strong antibacterial activity, a new clinical trial with Dermofural is being prepared to treat secondary bacterial infections in patients with diabetic foot.

In vivo G1 inhibits preferentially bacterial protein synthesis and *in vitro* tests have shown that the inhibition occurs at the level of initiator fMet-tRNA binding to the 30S subunit. Fast kinetics analyses indicate that, unlike the case of GE81112, which does not affect the formation of 30S pre-IC but inhibits its subsequent locking to form the 30S IC, G1 inhibits fMet-tRNA binding already at an early stage, blocking the formation of the 30S pre-IC (Figure 17.2 and Figure 17.5h). Remarkably, this inhibition displays a bias for the nature (purine vs pyrimidine) of the 3′ base of the codon, occurring efficiently only when the mRNA directing 30S IC formation and protein synthesis contains the canonical AUG initiation triplet or the rarely found AUA triplet but hardly occurs when the start codon is either one of the

noncanonical triplets AUU or AUC. This bias manifests itself only in the presence of IF3 and is of opposite sign compared to that displayed by IF3 itself; in fact, in its fidelity function, IF3 accepts the 30S IC formed with the canonical start codons (i.e., AUG, GUG, and UUG) and rejects those formed with the noncanonical triplets such as AUU and AUC [122].

It is interesting to note that kasugamycin (described earlier) also displays a differential inhibition of translation as a function of the nature of the initiation codon. In fact, at low concentrations, kasugamycin was found to allow relatively higher levels of β-galactosidase translation with 5′ pyrimidine start codons UUG and, even more so, CUG; although this effect was found to gradually disappear at higher concentrations of the antibiotic. At all concentrations of kasugamycin, the level of translation from the CUG initiation codon remained higher than that observed in its absence [94].

In situ cleavage experiments demonstrate that G1 influences essentially all the 16S rRNA bases implicated in P-site decoding and in IF3-dependent discrimination against noncanonical start codons. In fact, bases 1399 and 1404–1406, adjacent to the mRNA initiation codon, and bases 1336–1339, which constitute one side of the molecular gate separating P- and E-sites, are protected by G1, while bases 1387–1389, which have been implicated in codon discrimination by IF3 [11, 18, 21], and 1394, 1396, 1398 adjacent to the 3′-side of the mRNA initiation codon become more exposed. Finally, bases 1227 and 1229, which are near the N-terminal domain of S13, are also more exposed in the presence of the antibiotic (Figure 17.5i). Taken together, the finding that in the presence of G1 several nucleotides are protected and others more exposed to hydroxyl radicals gives a clear indication that, in addition to direct shielding effects, G1 induces conformational changes at specific 16S rRNA sites. Thus, it may be surmised that, as with IF3 [17, 19, 123], in the case of G1 also a ligand-induced conformational change of the 30S ribosomal subunit is at the root of initiation codon discrimination.

In light of the excellent knowledge of the 3D structure of 30S ribosomal subunit [e.g., 124] and of the fairly small size (MW 297) of G1, this antibiotic can be regarded as an interesting pharmacophore in the perspective of developing, through computational chemistry, rational design, or fragment-based drug design, a variety of efficient tools to fight bacteria, in particular those that have acquired multiple resistance to drugs targeting more common steps of translation [4, 125, 126].

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18
Inhibitors of Bacterial Elongation Factor EF-Tu

Attilio Fabbretti, Anna Maria Giuliodori, and Letizia Brandi

18.1
Introduction

Decoding of the genetic message occurs in a ribosomal site called *A-site*. Here, a given amino acid is specifically recognized by the anticodon triplet of a tRNA molecule carrying the corresponding amino acid. In all kingdoms of life, the aminoacyl-tRNA (aa-tRNA) does not bind to the ribosomal A-site in a free form but is always accompanied by an elongation factor (EF) with a bound guanosine-5′-triphosphate (GTP) molecule so that a ternary complex is the molecular form in which all aa-tRNAs, with the exception of the initiator tRNA, are brought to the ribosome. In bacteria, the EF responsible for this activity is EF-Tu, a notable representative of a widespread class of proteins called *G proteins*. The function of EF-Tu is not restricted to that of a simple carrier insofar as this factor plays, together with the ribosome, an active role in the faithful selection of the correct aa-tRNA. As with other members of the GTPase switch proteins, EF-Tu also undergoes important structural changes when its bound GTP molecule is hydrolyzed to GDP following the activation by the ribosome of the intrinsic GTPase activity of EF-Tu. Upon formation of codon–anticodon interaction, the affinity of EF-Tu·GDP for the ribosome is substantially decreased so that the factor can be dissociated from the ribosome. Unlike the other ribosome-dependent GTPases, IF2, and EF-G, which do not require a GTP–GDP exchange factor, GDP is dissociated from the EF-Tu and exchanged for GTP through the action of EF-Ts [1–3]. The translation elongation steps involved in the EF-Tu-dependent decoding of aa-tRNA are schematically represented in Figure 18.1.

EF-Tu is one of the major antibiotic targets among the protein synthesis inhibitors, a property that emphasizes its central role in protein synthesis and cell growth [4]. Four families of antibiotics of unrelated structures have EF-Tu as target. They comprise a total of more than 30 members and have selective antibacterial activities. Their prototypes are enacyloxin IIa, kirromycin, pulvomycin, and GE2270A.

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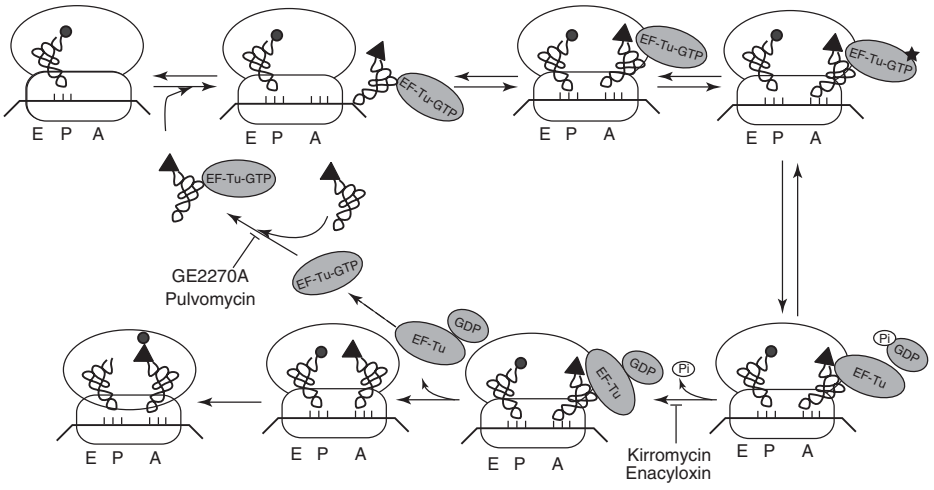

Figure 18.1 Scheme of the translation elongation steps involved in the EF-Tu-dependent decoding of aa-tRNA. In the scheme, the steps affected by enacyloxin, kirromycin, pulvomycin, GE2270A are indicated. More details on the molecular mechanism of A-site decoding and EF-Tu functioning can be found in the text and in [2, 4].

18.2 Enacyloxins

The family of related compounds known as *enacyloxins* was discovered during an antifungal screening program based on microbial interactions between soil strains and test organisms represented by several species of fungi [5]. The soil strain *Gluconobacter* sp. *W-315*, later classified as *Frateuria* sp. *W-315* [6], was identified as the producer of these unique polyenic antibiotics, which are effective against gram-positive and gram-negative bacteria but only somewhat active against fungi and inactive against yeasts [7].

These antibiotics can be obtained by fermentation of *Frateuria* sp. *W-325* in a spent medium of *Neurospora crassa* [7] or in a completely synthetic medium (modified Czapek-Dox medium), whose composition was defined for this purpose [8]. The isolation of enacyloxin IIa (MW 702 Da) led to the determination of its structure and stereochemistry by NMR spectroscopy, X-ray crystallography, sophisticated J-resolved heteronuclear multiple bond correlation (HMBC) analysis, and ketoreductase stereospecificity model [9–12]. It is a linear nonlactonic polyenic antibiotic consisting of 3,4-dihydroxycyclohexanecarboxylic acid with a chlorine-containing polyenic and polyhydroxy acyl side chain attached as an ester to the 3-hydroxyl substituent of the acid (Figure 18.2, structure **1**) [9]. Enacyloxin IIa is produced as an extracellular compound resulting from enzymatic oxidation of the precursor enacyloxin IVa [13]. Recently, it was discovered that enacyloxin IIa can be synthesized by *Burkholderia ambifaria* as a metabolic product of a cryptic modular polyketide synthase biosynthetic gene cluster [12]. Unlike usual lactonic polyenic

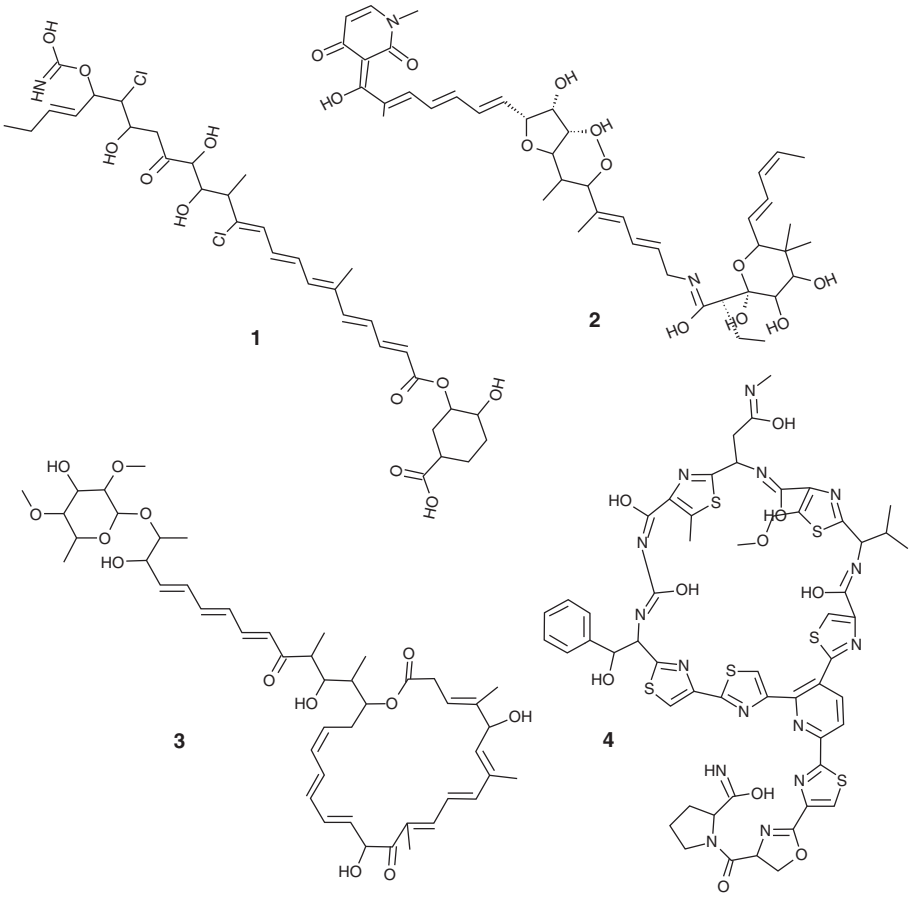

Figure 18.2 Chemical structures of the EF-Tu inhibitors described in this chapter. (**1**) Enacyloxin IIa, (**2**) kirromycin, (**3**) pulvomycin, and (**4**) GE2270A.

antibiotics that break down the cell membranes of yeast and fungi, the bacteriostatic effect of enacyloxin IIa stems from its ability to interfere with *in vivo* (inhibition of ^{14}C-amino acids into trichloroacetic-acid-insoluble fraction of *Escherichia coli K-12*) and *in vitro* protein synthesis (inhibition of poly(U) cell-free translation) [14]. Among the numerous targets involved in translation, enacyloxin IIa was found to specifically inhibit some of the functions of EF-Tu (Table 18.1). The studies aimed at the elucidation of the molecular mechanism of enacyloxin IIa action revealed that this antibiotic (i) impairs the binding of the aa-tRNA·GTP·EF-Tu complex to the A-site of ribosomes [15], (ii) does not affect the peptidyl-transferase center, and (iii) induces a distortion of the A-site-bound aa-tRNA, thereby preventing amino acid polymerization and translation elongation. A direct influence of the antibiotic on both EF-Tu and ribosomal A-site was reported [16], but it was later shown that the effect of enacyloxin IIa is restricted to EF-Tu [17]. This discovery was prompted by

Table 18.1 Effect of antibiotics on properties of EF-Tu.

Effects	Enacyloxin IIa	Kirromycin	Pulvomycin	GE2270A
Target	EF-Tu·GTP > EF-Tu·GDP	EF-Tu·GTP > EF-Tu·GDP	EF-Tu·GTP > EF-Tu·GDP	EF-Tu·GTP > EF-Tu·GDP
Binding properties	Transient interaction	Very stable interaction	Very stable interaction	Very stable interaction
EF-Tu affinity for GTP	Increased	Increased	Increased	Increased
EF-Tu affinity for GDP	Decreased	Decreased	Decreased	No effect
EF-Tu GTPase (intrinsic)	No effect	Increased	Increased	No effect
EF-Tu GTPase (ribosome dependent)	Increased	Increased	Inhibited	Inhibited
aa-tRNA binding	Increased for EF-Tu·GDP Decreased for EF-Tu·GTP	Increased for EF-Tu·GDP Decreased for EF-Tu·GTP	Inhibited	Inhibited
Protection of aa-tRNA from hydrolysis by EF-Tu	No	Yes	No	No
Electrophoretic mobility of native target–antibiotic complex	Increased with either GTP or GDP	Increased with either GTP or GDP	Increased with either GTP or GDP	Increased with either GTP or GDP
Effect on EF-Ts·EF-Tu interaction	Competition with EF-Ts for EF-Tu binding	Competition with EF-Ts for EF-Tu binding	Coexistence with EF-Ts on EF-Tu additive effects	No effect
Sensitivity dominant versus recessive	Sensitive dominant	Sensitive dominant	Sensitive dominant	Sensitive recessive

Table 18.2 Antibiotic-resistant EF-Tu mutants.

Antibiotic	Position	Substitution	Source of mutant EF-Tu
Kirromycin	120	Leu/Gln	*Salmonella enterica serovar typhimurium* [19]
	124	Gln/Arg, Glu	*Salmonella enterica serovar typhimurium* [19]
		Gln/Lys	*E. coli* [20]
	160	Tyr/Asn, Asp, Cys	*Salmonella enterica serovar typhimurium* [19]
	298	Ile/ΔIle	*Salmonella enterica serovar typhimurium* [19]
	316	Gly/Asp	*Salmonella enterica serovar typhimurium* [19], *E. coli* [20]
	329	Gln/His	*Salmonella enterica serovar typhimurium* [19]
	375	Ala/Ser, Thr, Val	*Salmonella enterica serovar typhimurium* [19]
		Ala/Thr, Val	*E. coli* [20]
	378	Glu/Lys	*E. coli* [20]
Enacyloxin IIa	124	Gln/Lys	*E. coli* [17]
	316	Gly/Asp	*E. coli* [17]
	375	Ala/Thr	*E. coli* [17]
Pulvomycin	230	Arg/Cys	*E. coli* [21]
	233	Arg/Ser	*E. coli* [22]
	230–233	Arg–Arg/Val–Phe	*E. coli* [22]
	333	Arg/Cys	*E. coli* [21]
	334	Thr/Ala	*E. coli* [21]
GE2270A	226	Val/Ala	*Bacillus subtilis* [67]
	257	Gly/Ser	*E. coli* [66]
	275	Gly/Ser	*Bacillus subtilis* [67]
		Gly/Ala	*E. coli* [66]

the findings that resistance to enacyloxin IIa can be obtained with EF-Tu mutants (in the presence of wt ribosomes) and when resistant and wt EF-Tu factors are mixed, both *in vivo* and *in vitro*, enacyloxin IIa sensitivity is dominant [17]. This latter effect can be explained only by supposing that in the presence of enacyloxin, sensitive EF-Tu (wt) can remain stuck on translating ribosomes regardless of the presence of resistant EF-Tu. Subsequently, using a preparation of ribosomes freed of all traces of EF-Tu, it was demonstrated that the only target of enacyloxin IIa is the EF [17]. Thus, as enacyloxin IIa and kirromycin (see subsequent text) have the same unique target and both display dominant sensitivity [17, 18], the two antibiotics were compared. The similarity between these two unrelated molecules was investigated by the use of kirromycin-resistant EF-Tu mutants, which were tested for their response to enacyloxin IIa. But for one mutation (A375V) that increases the EF-Tu sensitivity to enacyloxin IIa, all the other mutations located either in the domain 1–3 interface of *E. coli* EF-Tu (A375T, G316D, Q124K) or outside this interface (Q329H), confer resistance to both kirromycin and enacyloxin IIa (Table 18.2 and Figure 18.3) [17]. Taken together, the data obtained on the mechanism of action of enacyloxin IIa suggest that, similar to kirromycin, this antibiotic freezes EF-Tu in a sort of GTP-bound conformation within an A-site-bound aa-tRNA·GDP·EF-Tu complex, thereby preventing peptide bond formation (Figure 18.1) [17].

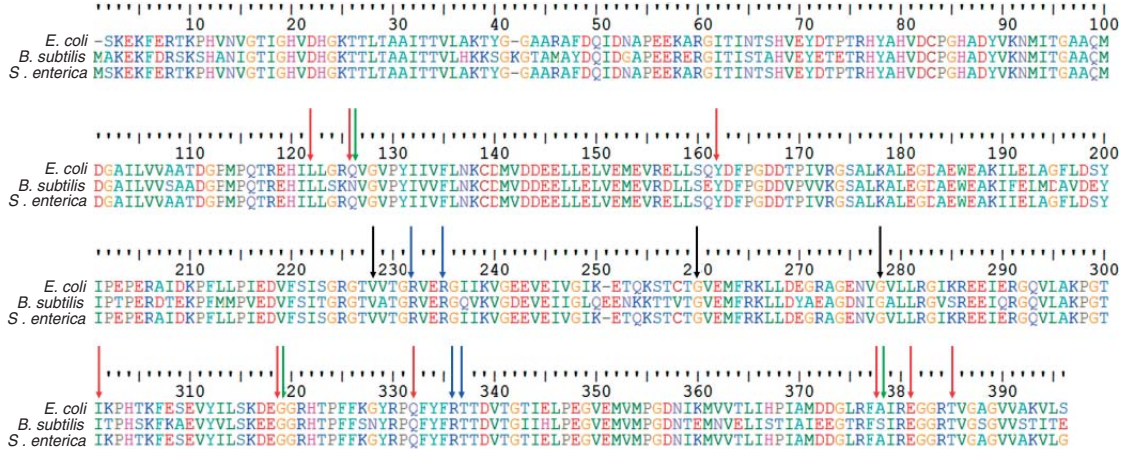

Figure 18.3 Locations of the mutations conferring antibiotic resistance within the primary sequence of *Escherichia coli*, *Bacillus subtilis*, and *Salmonella enterica* EF-Tu. Alignment of the amino acid sequence of *E. coli*, *B. subtilis*, and *S. enterica* EF-Tu in which the positions of the mutated amino acids conferring resistance to enacyloxin IIa (green arrows), kirromycin (red arrows), pulvomycin (blue arrows), and GE2270A (black arrows) are indicated.

The analysis of the EF-Tu mutants resistant to enacyloxin IIa suggests three molecular mechanisms of resistance: (i) modification of the enacyloxin IIa binding site of GTP·EF-Tu (Q124K, G316D) so that antibiotic–target interaction is impaired; (ii) lowered enacyloxin IIa affinity for ribosome-bound GDP·EF-Tu (Q124K, G316D, A375T) that allows antibiotic release from the target; and (iii) lowered enacyloxin IIa·GDP·EF-Tu (Q329H) affinity for aa-tRNA that allows the antibiotic–target release from the ribosomal A-site-bound aa-tRNA.

The crystal structures of the complexes enacyloxin IIa·GDPNP·*E. coli* EF-Tu (at 2.3 Å) and enacyloxin IIa·GDPNP·*Thermus aquaticus* EF-Tu·Phe-tRNA^{Phe} (at 3.1 Å) [10] have shed light on the similarities between enacyloxin IIa and kirromycin and suggested an explanation for the different phenotypes of the same A375V EF-Tu mutant *vis-à-vis* the two antibiotics. According to the structural analysis, enacyloxin IIa binds by an induced-fit mechanism at the interface of domains 1 and 3 of the factor, widens the interface, and reorients the side chain of several residues (Figure 18.4a,b) [10]. Kirromycin occupies the same site of the protein with a few differences, the most relevant of which is that the kirromycin tail is trapped in a hydrophobic pocket, whereas enacyloxin IIa passes along and outside the cavity (Figure 18.4a,b) [10]. This may explain not only the lower affinity of enacyloxin IIa for its target with respect to kirromycin [16] but also the different antibiotic response of the EF-Tu A375V mutant. In fact, EF-Tu becomes threefold more sensitive to enacyloxin IIa (it is stabilized) and about 300 times more resistant to kirromycin when Ala 375, which is situated close to the cavity, is replaced with a bulkier Val residue that fills the hydrophobic pocket so that the tail of the antibiotic can no longer fit into the cavity [10].

The structural studies also provided the interesting evidence that, as a consequence of the enacyloxin IIa binding to EF-Tu complex, the structure of the aa-tRNA is distorted so that the latter is no longer protected by the EF from spontaneous deacylation [10, 16]. Binding of the aa-tRNA to the target–antibiotic complex reverts the disorder induced by enacyloxin IIa on EF-Tu and is accompanied by a distortion of the tRNA acceptor stem. In any event, this structural alteration is compatible with the binding of enacyloxin·GTP·EF-Tu·aa-tRNA to the ribosomal A-site. Similar effects are reported for kirromycin [25].

A renewed interest in enacyloxin IIa was generated by the finding that this molecule possesses a potent activity against multidrug-resistant gram-negative pathogens such as *Burkholderia multivorans*, *Burkholderia dolosa*, and *Acinetobacter baumannii* [12], which are involved in respiratory infections associated with cystic fibrosis and other pathologies. Furthermore, as enacyloxin IIa is able to specifically inhibit the apicoplast EF-Tu of *Plasmodium falciparum* encoded by the apicoplast genome, it has been proposed that enacyloxin IIa, together with other EF-Tu inhibitors, could be used in an antimalarial therapy [26, 27].

To improve the anti-infective activity of enacyloxin IIa and to broaden its antibiotic application, it was suggested that its binding affinity be increased by constructing a chimera consisting of the head moiety of enacyloxin IIa and of the tail moiety of kirromycin [10].

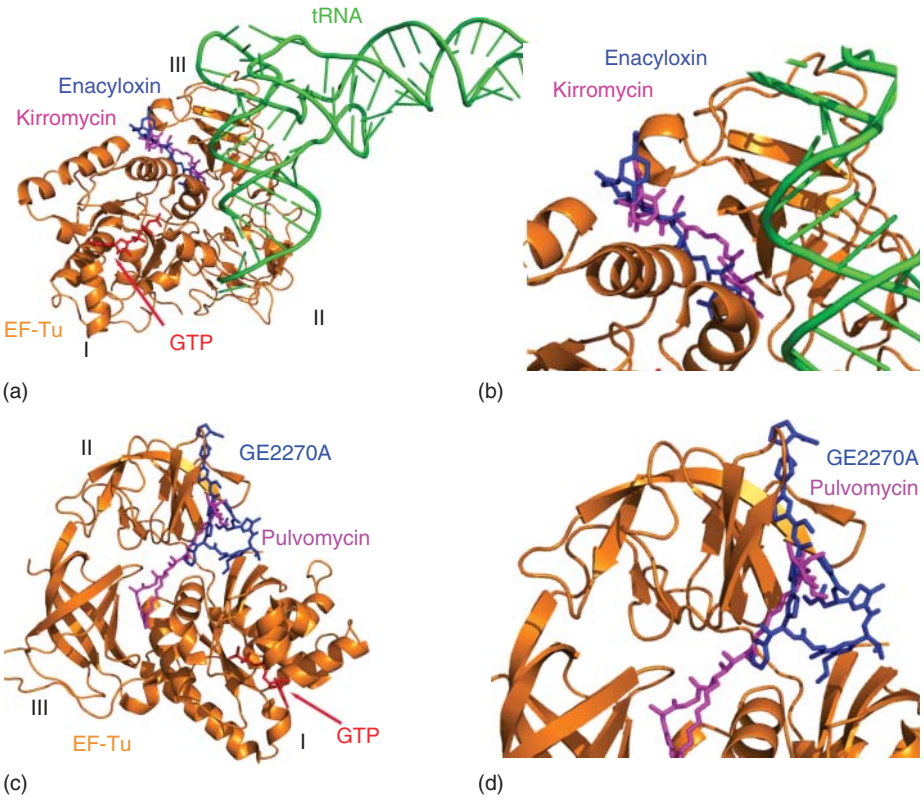

Figure 18.4 Three-dimensional structures of antibiotic-bound EF-Tu. (a) Structure of the complex of EF-Tu (ochre)·GTP (red)·Phe-tRNA (green) bound to enacyloxin (blue) (PDB file 1OB5) and kirromycin (magenta) (PDB file 1OB2). (b) Close-up of (a). (c) Structure of the complex of EF-Tu (ochre)·GTP (red) bound to GE2270A (blue) (PDB file 2C77) and pulvomycin (magenta) (PDB file 2C78). (d) Close-up of (c).

The structural information obtained from the crystallographic studies of the enacyloxin IIa-EF-Tu complex with or without aa-tRNA can be further exploited to rationally design additional, useful enacyloxin IIa modifications [10]. Finally, the recently proposed pathway for enacyloxin biosynthesis [12] could offer interesting possibilities to obtain variants of the antibiotic by genetic manipulations of its biosynthetic pathway.

18.3 Kirromycin

Kirromycin (MW 796.94 Da) is a linear polyketide containing three intramolecular ring systems: the pyridone ring, the central tetrahydrofurane moiety, and a sugarlike structure named goldinonic acid (Figure 18.2, structure **2**).

Kirromycin was the first antibiotic identified against EF-Tu and the best studied (for a review, see [18] and [28]). This molecule belongs to a group, named elfamycins, comprising at least 13 analogs, and was first isolated by Wolf and Zähner [29] from the actinomycete *Streptomyces collinus Tü 365*, in a screening program aimed at identifying narrow-spectrum antibiotics. Around that time, a compound identical to kirromycin named mocimycin and a 1-*N*-methyl derivative of kirromycin named aurodox were isolated from *Streptomyces ramocissimum* and from *Streptomyces goldiniensis*, respectively [30, 31]. Other microbial natural products structurally related to kirromycin are kirrothricin, efrotomycin, ganefromycin, heneicomycin, A83016F, factumycin, L-681,217, UK-69,753, phenelfamycins, and azdimycin.

The production of these antibiotics is characterized by a low fermentation yield, apparently due to a regulation by feedback inhibition [32] that is a consequence of the binding of the antibiotic to the *Streptomyces* EF-Tu, which is antibiotic sensitive. The yield of the antibiotic was considerably improved on introduction of kirromycin or kirromycin-like resistant strains [33].

The kirromycin-like antibiotics are narrow-spectrum antibacterial agents and the activity of most of them (mocimycin, aurodox, efrotomycin, heneicomycin, and kirrothricin) was found to be similar [34, 35]. Aurodox primarily acts against gram-positive bacteria, and also, albeit usually to a lesser extent, against certain gram-negative bacteria [31]. Kirromycin was found to be also active against certain gram-positive and gram-negative bacteria, whereas all actinomycetes were moderately to highly sensitive to this antibiotic [29].

Previous studies proved that kirromycin has strong activity against *Streptococci*, some strains of *Enterococci*, *Neisseria gonorrhoeae*, and *Haemophilus influenzae* [36], whereas *Staphylococcus aureus* is intrinsically resistant to the antibiotic owing to the presence of a kirromycin-resistant EF-Tu in this organism [37]. Efrotomycin does not inhibit cell-free protein synthesis in cell extracts from *Bacillus subtilis*, *S. aureus*, or *Enterococcus faecalis*, which are resistant to this antibiotic. Also, the cell-free translation systems from Streptococci are somewhat less sensitive to efrotomycin than those derived from gram-negative bacteria. Therefore, naturally occurring resistance to kirromycins in gram-positive bacteria seems to be mediated by their antibiotic-insensitive EF-Tu in which the kirromycin-binding site is less conserved than that of gram-negative species (Table 18.2 and Figure 18.3) [38]. Two main causes account for this narrow susceptibility range of kirromycin in the tested organisms. Mutations in the EF-Tu (*tuf*) gene give rise to EFs that are modified around the kirromycin-binding site, yet fully functional in protein synthesis and resistant to the antibiotic (e.g., [19, 20], and Table 18.2). In other cases, it has been suggested that kirromycin and related compounds cannot penetrate the cells or are pumped out of the cells before their interaction with EF-Tu. In fact, while EF-Tu insensitivity accounts for kirromycin resistance in *S. aureus* [37], in *E. coli* lack of membrane permeability is the critical factor that renders these cells insensitive to kirromycin despite having a kirromycin-sensitive EF-Tu [39, 40]. Kirromycin is active against the apicoplast EF-Tu of the malaria parasite *P. falciparum*, interferes with *in vitro* organelle-based protein biosynthesis, and is active against blood cultures of this protozoan [26, 27]. Because no toxic effects of

kirromycin or kirromycin-like antibiotics were observed in higher eukaryotic cells [41], these antibiotics represent potential weapons in the antimalarial battle.

The mechanism of action of kirromycin is similar to that observed for the enacyloxin IIa. Kirromycin binding at the interface of domains 1 and 3 of EF-Tu (Figure 18.4a,b) in the EF-Tu·GDP complex induces the factor to assume a GTP-like conformation, despite the presence of bound GDP [25]. Indeed, the induction by kirromycin of a GTP conformation in EF-Tu·GDP [42] causes this complex to stick to the mRNA-programmed ribosome after aa-tRNA binding and inhibits the release of the EF-Tu·GDP·antibiotic complex from the ribosome after GTP hydrolysis. From this ensues a blockage of the formation of the next peptide bond (Figure 18.1) [42, 43].

The GTP-like conformation of EF-Tu·GDP·kirromycin explains why this complex shares functional properties with the antibiotic-free ''on''-form, such as the formation of a stable complex with aa-tRNA, that binds to the programmed ribosome [42, 43].

18.4 Pulvomycin

Pulvomycin (MW 839 Da) was first identified as a *Streptomyces* product [44]. Shortly thereafter, labilomycin, a very labile antibiotic, was also discovered [45] and later on these two molecules were found to be the same antibiotic [46] produced as intramycelium products of *Streptoverticillium netropsis* and *Streptomyces albosporeus* var. *labilomyceticus*. The partial structure of pulvomycin [47] led to the notion that this antibiotic was related to the 5′ substituent of the central tetrahydrofuran of kirromycin [48], but when the pulvomycin structure was completed [49] it was found to be considerably more complicated, being composed of a 22-membered lactone ring with a side chain formed by two trienones, one triene, and a terminal sugar (Figure 18.2, structure **3**). The microbiological activity of pulvomycin is mainly directed against gram-positive bacteria and is negligible versus gram-negative bacteria [50, 51]. In a recent study, pulvomycin was produced by an engineered strain of *Streptomyces flavopersicus* and showed antibacterial activities never reported in the past against highly antibiotic resistant pathogens such as *Burkholderia cenocepacia*, *Burkholderia vietnamiensis*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* as well as clinical isolates of methicillin-resistant *S. aureus* and vancomycin-resistant *Enterococcus* (*vanB*) [52].

Pulvomycin activity was also demonstrated against a variety of malignant cells in tissue culture and against Ehrlich carcinoma cells in mice [45, 46] but not against nontransformed eukaryotic cells [53]. Although pulvomycin is active against both bacteria and malignant eukaryotic cells, the molecular target of the inhibition is different in the two kingdoms of life: in bacteria pulvomycin inhibits protein synthesis elongation interfering with EF-Tu function [48], whereas in eukaryotic cells it inhibits RNA synthesis [41]. Despite the fact that pulvomycin is not active against some gram-negative bacteria, EF-Tu extracted from gram-negative

organisms is even more sensitive to pulvomycin inhibition than that extracted from gram-positive organisms [51]. This finding indicates the existence of an antibiotic penetration problem in the gram-negative cells. In addition to eubacterial EF-Tu, pulvomycin is active also against some archaebacterial EF-Tu equivalents, suggesting that the pulvomycin-binding site might be an ancient trait already present in the EF of an ancestral form of life [54].

The molecular mechanism of pulvomycin action was investigated in depth ([55] and references therein) and some effects of this antibiotic on EF-Tu are reported in Table 18.1. Pulvomycin binds to EF-Tu and increases a 1000 times the stability of the EF-Tu·GTP complex by causing a highly diminished GTP dissociation rate. In contrast, it increases 25-fold the GDP dissociation rate, resembling the action of EF-Ts. Binding of the latter factor and of pulvomycin to EF-Tu can coexist and their effects on EF-Tu are additive.

Similar to kirromycin, pulvomycin enhances the intrinsic EF-Tu GTPase activity, depending on the nature and concentration of monovalent cations, but in contrast to kirromycin, pulvomycin inhibits the EF-Tu GTPase in the presence of aa-tRNA and ribosomes (Figure 18.1) [48, 55]. Pulvomycin is reported to have a protective effect on EF-Tu·GTP against urea-induced denaturation and tryptic digestion and to facilitate urea denaturation and proteolytic cleavage of EF-Tu·GDP. Taken together, the pulvomycin effects on EF-Tu indicate that this antibiotic interferes with the allosteric control of EF-Tu conformation, stabilizing an anomalous EF-Tu·GTP-bound state that is unable to form the ternary complex with the aa-tRNA [48, 55]. In turn, this prevents aa-tRNA delivery to the ribosomal A-site (Figure 18.1). In particular, crystallographic studies have shown that pulvomycin binds, by an induced-fit mechanism, at the three domains junction of EF-Tu (Figure 18.4c,d), freezing the EF-Tu·GTP in a GDP-like ''off-state'' [42] by widening and reorienting the domain 1-2-3 junction, thereby changing the juxtaposition of domains 1 and 2 to avoid steric clash of domain 1 with the antibiotic [56]. Pulvomycin binding also affects switch I (*E. coli* EF-Tu D51—T64) and switch II (*E. coli* EF-Tu G83—A95) regions of the factor, which mediate the EF-Tu conformational changes and the GTPase activity ([2, 4] and references therein). The stable binding of pulvomycin to EF-Tu is obtained by extensive hydrophobic interactions resembling the binding of GE2270A (see subsequent text) with which it partially overlaps on EF-Tu [56]. In particular, from their positions on the target, both pulvomycin and GE2270A interfere with the binding of the 3′ aminoacyl group and the acceptor stem of the aa-tRNA, and pulvomycin (but not GE2270A) hinders the binding of the 5′ end of the aa-tRNA [56]. The amino acids whose substitutions confer pulvomycin resistance, that is, Arg230Cys, Arg230Val/Arg233Phe, Arg 233Ser/Cys, Thr334Ala (Table 18.2 and Figure 18.3), are located along the binding surface [22, 56, 57]. These mutations alter the pulvomycin-binding site but allow protein synthesis to occur both *in vivo* and *in vitro* [22, 57]. Interestingly, similar to that of kirromycin and enacyloxin IIa and unlike that of GE2270A, pulvomycin sensitivity is dominant over resistance with EF-Tu mutants [17, 18, 21, 22, 58]. The accepted mechanism of action for kirromycin and enacyloxin IIa, by which they may block sensitive EF-Tu on the ribosome, can be easily reconciled with the observed sensitive dominance.

However, in the case of pulvomycin, the inhibition of protein synthesis by substrate limitation [48] may hardly account for the reported dominance of sensitive EF-Tu and suggests that an additional mechanism must block translation rather than a simple aa-tRNA limitation [22, 59].

18.5 GE2270A

GE2270A (MW 1297 Da) is a member of the cyclic thiazolyl peptide family (Figure 18.2, structure **4**) and structurally related to thiostrepton and micrococcins, which are also inhibitors of protein synthesis and act by targeting directly the ribosome. GE2270A is produced as a complex of 10 structurally related compounds during the exponential growth phase of *Planobispora rosea* ATCC53773 [50], a member of a rare genus of actinomycetes [60, 61]. The addition of increasing concentrations of vitamin B12 to the *P. rosea* fermentation medium exerted two significant effects on GE2270 productivity [62]. Total complex productivity was doubled by the addition of vitamin B12 in the range 0.001–0.01 mg ml^{-1}. A further effect was an increase in the synthesis of GE2270 component A; the relative abundance of this component increased from 60% to >90% on the addition of vitamin B12 to the standard fermentation conditions.

GE2270A has excellent activity against gram-positive bacteria, with minimum inhibitory concentrations (MICs) ~0.1 µg ml^{-1} for *Staphylococci* and *Enterococci*. GE2270A was as active against isolates resistant to ampicillin, methicillin, erythromycin, glycopeptides, and gentamicin as against susceptible bacteria. GE2270A has weak (with *Enterococci*) to moderate (with *Staphylococci*) bactericidal activity. Nevertheless, it has excellent activity against staphylococcal and streptococcal septicemia in mice and against staphylococcal and enterococcal endocarditis in rats [63].

The GE2270A *P. rosea* producing strain is constitutively resistant to this antibiotic. The resistance of the producing strain is mediated by a conspicuous number of mutations of conserved amino acid residues of EF-Tu, which render the EF GE2270A insensitive [58, 61, 64]. In addition, the specialized EF-Tu3 from *Streptomyces ramocissimus* (kirromycin producer strain) was also found to be GE2270A resistant as a result of similar amino acid changes [65].

The GE2270A-binding site differs from that of pulvomycin and comprises three EF-Tu segments, including residues 215–230, 256–264, and 273–277 (Figure 18.4c,d). The binding sites of the two antibiotics only share 7 amino acids out of more than 20 contacted by the two molecules on EF-Tu.

GE2270A binds to domain 2 of EF-Tu, the upper part of its thiazolyl ring contacting domain 1 in EF-Tu·GDPNP (Figure 18.4c,d) [56]. In contrast, in EFTu·GDP the upper part of the thiazolyl ring is exposed to the solvent as a consequence of the conformational change induced by GDP on EF-Tu [64].

In *E. coli*, two EF-Tu mutations (G257S and G275A) confer high GE2270A resistance (Table 18.2) [24]. In addition, two mutations (V226A and G275S) causing resistance to GE2270A have been identified in *B. subtilis* EF-Tu (Table 18.2)

[61, 23]. These residues are located in domain 2, close to the binding site for the 3′ end of aa-tRNA [66].

Similar to pulvomycin, the inhibition of protein synthesis by GE2270A appears to stem from its ability to inhibit the formation of the EF-Tu·GTP·aa-tRNA ternary complexes (Figure 18.1) [67]. As seen in Table 18.1, when GE2270A binds to EF-Tu, its affinity for GTP is strongly increased, while that for GDP remains unaltered, so that only the functions of the EF-Tu·GTP complex are affected. These observations, along with studies demonstrating that GE2270A resistance is dominant over sensitivity [21, 58], suggest that its mode of action may be distinct from that of pulvomycin.

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19
Aminoglycoside Antibiotics: Structural Decoding of Inhibitors Targeting the Ribosomal Decoding A Site

Jiro Kondo and Eric Westhof

19.1
Introduction

Decoding is the initial process of protein synthesis and occurs at the aminoacyl-tRNA binding site (the A site) on the small ribosomal subunit (Figure 19.1a). The A site has an RNA molecular switch composed of 15 nucleotide residues in which the consecutive adenines A1492 and A1493 in an asymmetrical internal loop are essential for the discrimination of cognate tRNAs from near-cognate tRNAs (Figure 19.1b) [1–4]. In the absence of aminoacyl-tRNA, the molecular switch is conformationally dynamic and the two adenines adopt various conformations [5]. These variable states are called *off*. When an aminoacyl-tRNA is delivered to the A site, the two adenines flip out from the A-site internal loop and participate in the recognition of the first two base pairs of the codon–anticodon mini-helix through A-minor interactions to check whether these base pairs are of the canonical Watson–Crick type or not (Figure 19.1b,c). This unique state is called *on*. The switching of the A site from the ''off'' to ''on'' states provokes a global conformational change of the 30S ribosome from an open (empty) to a closed (tRNA-bound) form that leads to transpeptidation and translocation (Figure 19.1a) [1–4].

Aminoglycosides are decoding inhibitors that target the bacterial A-site molecular switch and display broad-spectrum antibacterial activity against a variety of gram-negative and certain gram-positive bacteria [6–12]. The structural basis for their antibacterial activity was initially revealed by an X-ray analysis of the 30S ribosome in complex with paromomycin solved at 3.0 Å resolution by Ramakrishnan's group [13]. At the same period, Vicens and Westhof [14] determined the interaction mode of paromomycin to the A site at a better resolution (2.5 Å) by using an RNA oligomer designed to be folded as a double helix containing the two A sites. As the packing interaction observed in the oligomer-based crystal perfectly mimics the A-minor interactions between the A site and codon–anticodon mini-helix occurring in the ribosome, the model system as well as other RNA oligomers have been extensively used to determine interaction modes of several aminoglycosides to the A site at high resolution by Westhof's and Hermann's groups [15–27]. A series of X-ray analyses has confirmed that aminoglycosides specifically bind to the bacterial A-site

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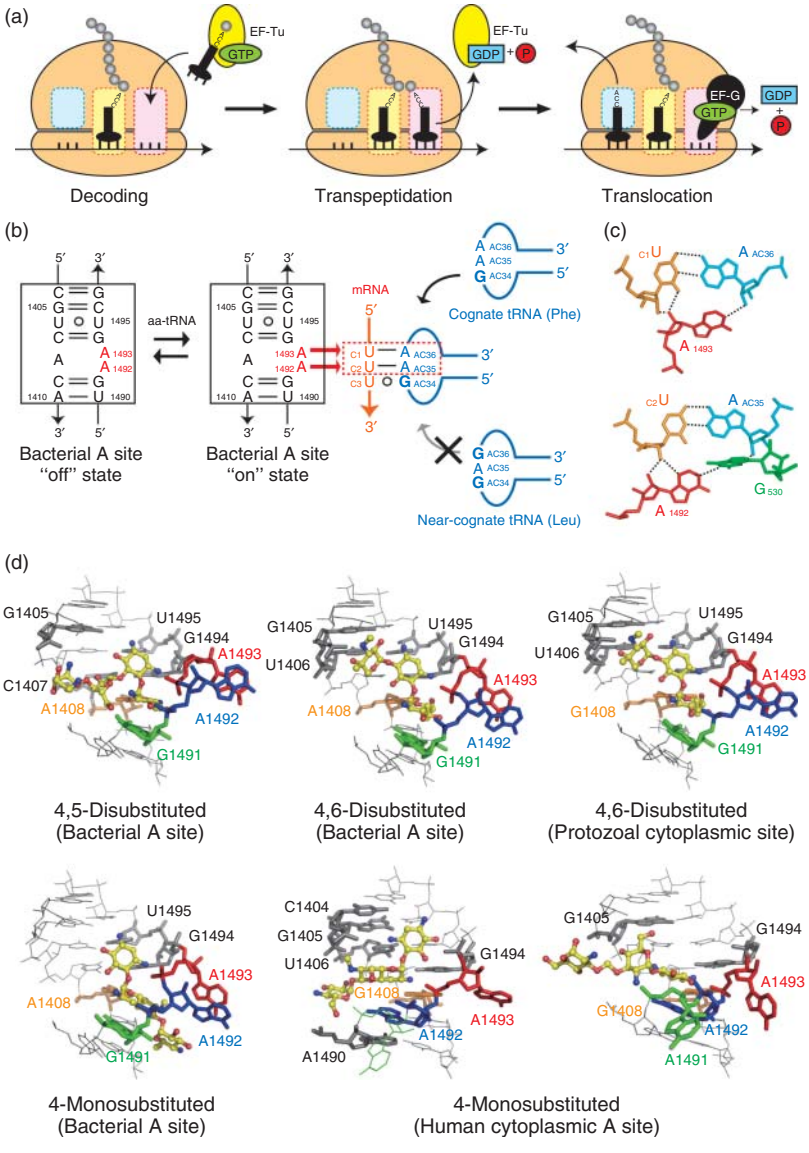


Figure 19.1 (a) Schematic diagram of decoding, transpeptidation, and translocation processes in protein biosynthesis. (b) Schematic diagram of the A-site molecular switch that discriminates between the cognate and near-cognate tRNAs. (c) The A-minor recognitions of the first two Watson–Crick base pairs in the codon–anticodon mini-helix by the three bases A1492, A1493 (from the A-site molecular switch on helix 44), and G530 (from helix 18). (d) Specific binding of aminoglycosides to the bacterial, protozoal cytoplasmic, and human cytoplasmic A-site molecular switches. Nucleotide residues interacting with aminoglycosides are labeled. EF-Tu, elongation factor thermo unstable.

molecular switch and locks it in the "on" state. Therefore, the ribosome loses the ability to discriminate between cognate and near-cognate tRNAs. Aminoglycosides are known to induce conformational changes of the 16S rRNA so that tRNAs bind with higher affinity to the A site [28]. A recent study [29] concluded that the effects of aminoglycoside binding to the ribosome are not restricted to forcing A1492/A1493 in the bulged-out conformation but that, upon binding, aminoglycoside alter, and provoke a relaxation of the whole decoding pocket, leading to a higher affinity for near-cognate tRNAs.

Miscoding should also occur in eukaryotes if aminoglycosides bind to eukaryotic A sites. Some aminoglycosides display antiprotozoal activity, and the molecular mechanism has recently been cleared [30]. In addition, some of them are toxic to mammals. Although structural evidence is rather poor compared to the antibacterial and antiprotozoal activities, the toxicity to the human ear and kidney cells resulting from the clinical use of aminoglycosides has been considered to originate from the binding of these drugs to the human mitochondrial and cytoplasmic A sites [9, 10, 31–35].

In this chapter, the molecular recognition of aminoglycosides to the ribosomal A-site molecular switches are reviewed, and structural bases for their antibacterial and antiprotozoal activities and toxicity to humans are discussed together with that for antibiotic resistance against aminoglycosides. The comparative analyses of the interactions observed between aminoglycosides and the A site offer insights into the molecular recognition that is helpful for structure-based drug design.

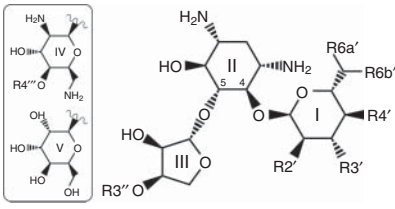
19.2
Chemical Structures of Aminoglycosides

Aminoglycosides are positively charged oligosaccharides composed of a variable number of sugar rings containing several ammonium (NH_3^+) and hydroxy (OH) groups, and have a 2-deoxystreptamine (2-DOS or ring II) ring in common. The A-site inhibitors can be classified according to the chemical linkages between the central ring II and the attached rings: 4,5-disubstituted, 4,6-disubstituted, and 4-monosubstituted classes. Each class can be further divided into two subclasses according to a functional group attached to position 6′ on ring I: 6′-NH_3^+ and 6′-OH subclasses (Figure 19.2 and Table 19.1). The difference between these subclasses is critical to their pharmacological activity. Aminoglycosides belonging to the latter subclass tend to exhibit activity against certain antibiotic-resistant bacteria and parasitic protozoa (Table 19.1) [10].

19.3
Secondary Structures of the Target A Sites

Secondary structures of the A-site molecular switches are highly conserved in nature (Figure 19.3). The RNA molecular switch is composed of 15 nucleotide

(a) 4,5-Disubstituted class



6′-NH_3^+ subclass

Neomycin B:
(Ring I) R2′ = NH_3^+, R3′ = OH, R4′ = OH, R6a′ = NH_3^+, R6b′ = H
(Ring III) R3″ = Ring IV with R4‴ = H

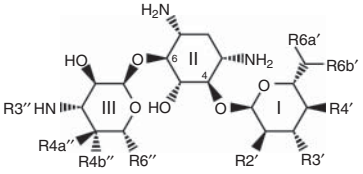
Ribostamycin:
(Ring I) R2′ = NH_3^+, R3′ = OH, R4′ = OH, R6a′ = NH_3^+, R6b′ = H
(Ring III) R3″ = H

6′-OH subclass

Paromomycin:
(Ring I) R2′ = NH_3^+, R3′ = OH, R4′ = OH, R6a′ = OH, R6b′ = H
(Ring III) R3″ = Ring IV with R4‴ = H

Lividomycin A:
(Ring I) R2′ = NH_3^+, R3′ = H, R4′ = OH, R6a′ = OH, R6b′ = H
(Ring III) R3″ = Ring IV with R4‴ = Ring V

(b) 4,6-Disubstituted class



6′-NH_3^+ subclass

Gentamicin C1a:
(Ring I) R2′ = NH_3^+, R3′ = H, R4′ = H, R6a′ = NH_3^+, R6b′ = H
(Ring III) R3″ = CH_3, R4a″ = CH_3, R4b″ = OH, R6″ = H

Tobramycin:
(Ring I) R2′ = NH_3^+, R3′ = H, R4′ = OH, R6a′ = NH_3^+, R6b′ = H
(Ring III) R3″ = H, R4a″ = OH, R4b″ = H, R6″ = CH_2OH

Kanamycin A:
(Ring I) R2′ = OH, R3′ = OH, R4′ = OH, R6a′ = NH_3^+, R6b′ = H
(Ring III) R3″ = H, R4a″ = OH, R4b″ = H, R6″ = CH_2OH

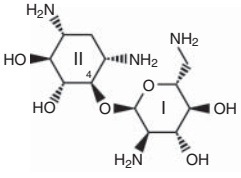
Kanamycin B:
(Ring I) R2′ = NH_3^+, R3′ = OH, R4′ = OH, R6a′ = NH_3^+, R6b′ = H
(Ring III) R3″ = H, R4a″ = OH, R4b″ = H, R6″ = CH_2OH

6′-OH subclass

Geneticin (G418):
(Ring I) R2′ = NH_3^+, R3′ = OH, R4′ = OH, R6a′ = OH, R6b′ = CH_3
(Ring III) R3″ = CH_3, R4a″ = CH_3, R4b″ = OH, R6″ = H

(c) 4-Monosubstituted class

6′-NH_3^+ subclass (neamine)



6′-OH subclass (apramycin)

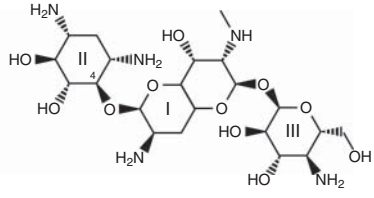


Figure 19.2 Chemical structures of aminoglycosides; (a) 4,5-disubstituted, (b) 4,6-disubstituted, and (c) 4-monosubstituted classes.

Table 19.1 Minimum inhibitory concentrations (μg ml^{-1}) of aminoglycosides.

Aminoglycosides	A1408		G1408		
	Bacteria wild-type	Human mitochondria[a]	Bacteria A1408G	Protozoa cytoplasm[b]	Human cytoplasm[b]
4,5-Disubstituted 6′-NH_3^+					
Neomycin B	1–2 (2ET4/2.4 Å) (2A04/3.0 Å)	16–32	>1024	>1024	>1024
Ribostamycin	8 (2ET5/2.2 Å)	—	>1024	—	—

Table 19.1 (*Continued*)

Aminoglycosides	A1408		G1408		
	Bacteria wild-type	**Human mitochondria[a]**	**Bacteria A1408G**	**Protozoa cytoplasm[b]**	**Human cytoplasm[b]**
4,5-Disubstituted 6′-OH					
Paromomycin	1 (1J7T/2.5 Å)	>1024	64	128	≥1024 $IC_{50} = 57\,\mu M$[c]
Lividomycin A	2 (2ESJ/2.2 Å)	—	64	—	—
4,6-Disubstituted 6′-NH_3^+					
Gentamicin C1A	1 (2ET3/2.8 Å)	64–128	>1024	>1024	>1024
Tobramycin	2 (1LC4/2.5 Å)	128	>1024	1024	1024
Kanamycin A	0.5–1 (2ESI/3.0 Å)	256–512	>1024	>1024	>1024
Kanamycin B	2–4	—	>1024	—	—
Amikacin	1 (2G5Q/2.7 Å)	32–64	>1024	—	—
4,6-Disubstituted 6′-OH					
Geneticin (G418)	16–32 (1MWL/2.4 Å)	—	64–128 (3TD1/2.1 Å)	4 (3TD1/2.1 Å)	128
4-Monosubstituted 6′-NH_3^+					
Neamine	64 (2ET8/2.5 Å)	—	>1024	—	—
4-Monosubstituted 6′-OH					
Apramycin	5[d] $IC_{50} = 0.05\,\mu M$[c] $K_d = 2.0\,\mu M$[e] (1YRJ/2.7 Å)	—	>1280[d]	—	— $IC_{50} = 29\,\mu M$[c] $K_d = 0.5\,\mu M$[e] (2G5K/2.8 Å)
NB33	96[f] $IC_{50} = 1.1\,\mu M$[c]	—	—	—	— $IC_{50} = 2.4\,\mu M$[c] (2O3V/2.8 Å)

PDB-IDs of X-ray structures with the best resolution are in parentheses.
Not marked, taken from [6, 7].
[a] Taken from [9].
[b] Taken from [10].
[c] The half maximal concentrations taken from [33].
[d] Taken from [8].
[e] Dissociation constants taken from [11].
[f] Taken from [12].

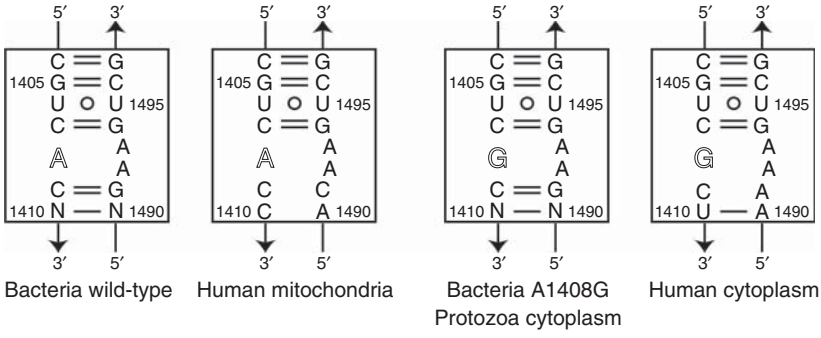


Figure 19.3 Secondary structures of the A-site molecular switches. The rRNA residues are numbered according to *E. coli* nomenclature.

residues. At the upper side of the switch, nucleotide sequences are conserved, and three Watson–Crick G=C and a bifurcated UoU base pairs are formed. At the center of the A-site helix, three residues construct an asymmetrical internal loop. Two of them, the 1492 and 1493 residues, are universally conserved adenines that can adopt various conformations, allowing the A site to function as a molecular switch. The 1408 residue on the opposite strand is an adenine in bacteria and eukaryotic mitochondria and a guanine in eukaryotic cytoplasm small ribosomal particles. The bottom of the A-site switch (base pair 1409–1491) is more variable than the other parts. The variability of the 1408 residue and the bottom part of the A site is linked to susceptibility to aminoglycosides (Table 19.1). In general, the bacterial and eukaryotic mitochondrial A sites with A1408 are more susceptible to aminoglycosides compared to the cytoplasmic A site with G1408. Bacteria acquire resistance against aminoglycosides by an A1408G spontaneous mutation in 16S rRNA at chromosomal level without losing their fitness [36, 37]. The bacterial and protozoal A sites with the canonical Watson–Crick base pairs between the 1409 and 1491 residues are more susceptible to aminoglycosides compared to the human mitochondrial and cytoplasmic A sites with a mismatch at the same position. The selectivity of aminoglycosides allows for their use as therapeutic agents for several bacterial infections.

19.4 Overview of the Molecular Recognition of Aminoglycosides by the Bacterial A Site

It has been revealed by X-ray analyses that the 4,5- and 4,6-disubstituted and 4-monosubstituted aminoglycosides specifically bind to the ''on'' state of the bacterial A site through several conserved contacts (Figure 19.1d) [13–27]. These three types of aminoglycosides have rings I and II in common. Ring I is inserted into the A-site helix by stacking on G1491 and forms a Watson–Crick pseudo pair with A1408. Ring II interacts with four consecutive residues on the long strand, A1492, A1493, G1494, and U1495. Extra rings of the 4,5- and 4,6-disubstituted aminoglycosides

interact with atoms of G1405, U1406, and C1407 on the short strand. It is obvious from these observations that rings I and II contribute to binding specificity and that the other rings increase binding affinity to the A site.

19.5
Role of Ring I: Specific Recognition of the Binding Pocket

During the decoding process, the A site dynamically changes its conformation and a deep pocket is created between G1491 and G1494 when the switching residues A1492 and A1493 bulge out. The pocket is specifically recognized by ring I. The sugar ring I with a chair conformation, inserted into the pocket, stacks on the G1491 residue through CH/π interactions (Figure 19.1d). The characteristic ring I of the 4,6-disubstituted aminoglycoside sisomicin with a C4′=C5′ double bond stacks on G1491 by sharing their π-electron densities [38]. Simultaneously with the stacking interaction, ring I forms a Watson–Crick pseudo pair with the A1408 residue. The pseudo-pair geometries are conserved between 6′-NH_3^+ and 6′-OH subclasses (Figure 19.4a–c). Two hydrogen bonds O5′ . . . H–$N6_{A1408}$ and O6′/N6′–H . . . $N1_{A1408}$ are observed in the pseudo pairs.

On the other side of ring I, NH_3^+ and OH groups are attached at positions 2′, 3′, and 4′. Ring I can be classified into four types according to the functional groups attached at these positions (Figure 19.4e). Position 2′ has either an NH_3^+ or an OH group, but the functional group is not used for direct A-site recognition. The group at position 2′ is hydrated and contributes either to the overall charge of the antibiotic or to the basicity of the neighboring groups [39]. On the other hand, OH groups at positions 3′ and 4′ make hydrogen bonds to the phosphate oxygen atoms of A1492 and A1493, thereby stabilizing these adenines in the bulged-out conformations.

The inactivation of aminoglycosides by enzymes, *N*-acetyltransferases (AAC), *O*-nucleotidyltransferase (ANT), and *O*-phosphotransferases (APH), are the most prevalent and clinically relevant antibiotic-resistance mechanism against aminoglycosides [40]. Each enzyme acetylates, adenylates, or phosphorylates a functional group attached to the specific position by using cofactors acetyl-CoA or ATP. The functional groups of ring I that are critical for the specific A-site recognition, 6′-NH_3^+/OH, 3′-OH, and 4′-OH groups, can be modified by AAC(6′), ANT(6′), ANT(3′), APH(3′), and ANT(4′), respectively.

19.6
Role of Ring II (2-DOS Ring): Locking the A-Site Switch in the "On" State

The chemical structure and the interaction mode of the central ring II are conserved (Figure 19.4f). The N1 atom makes a hydrogen bond to the O4 atom of U1495. The N3 atom makes three hydrogen bonds N3–H . . . $N7_{G1494}$, N3–H . . . $O2P_{G1494}$, and N3–H . . . $O1P_{A1493}$. The latter two interactions force to maintain the "on" state with the bulged-out A1492 and A1493 regardless of whether the codon–anticodon stem is cognate or near-cognate type, which contributes to misreading of the

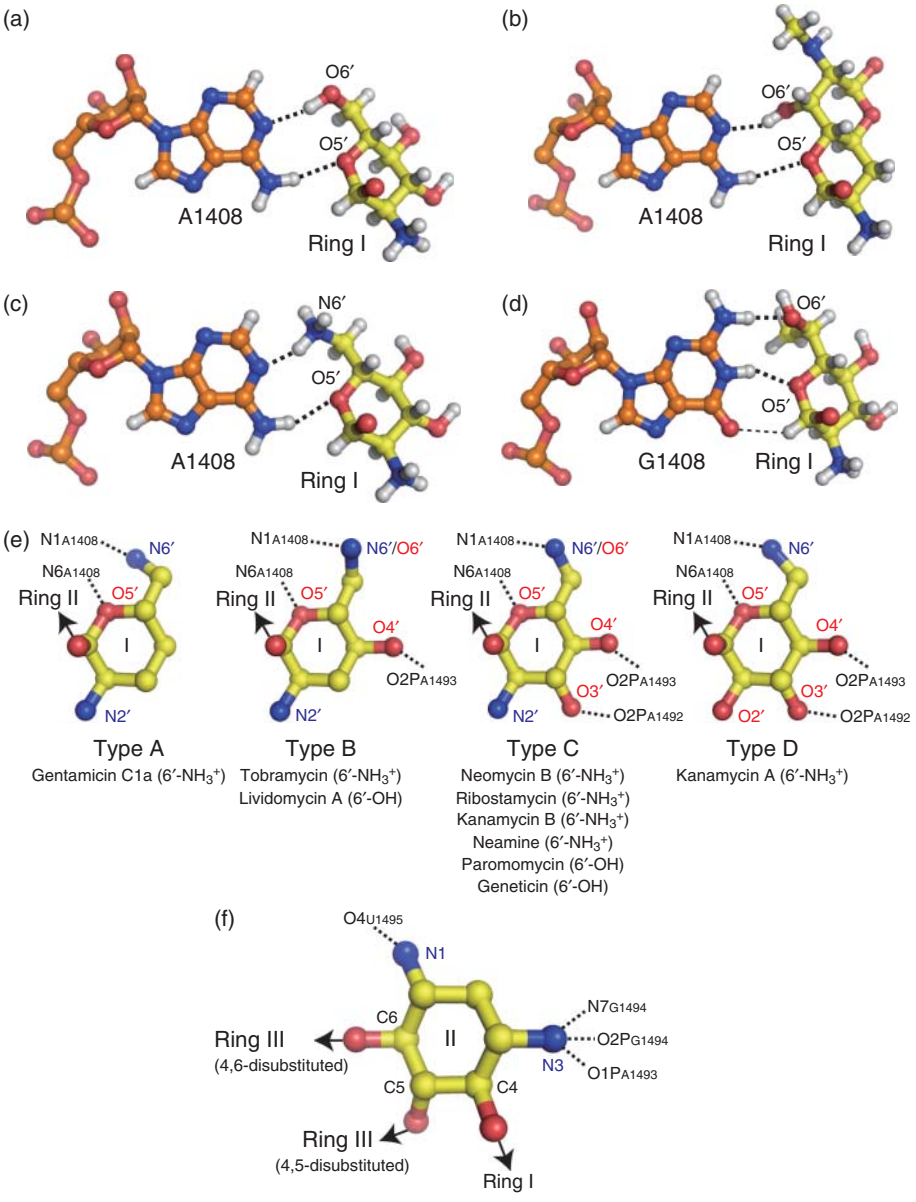


Figure 19.4 (a) Pseudo pairs between ring I containing a 6′-OH group and A1408, (b) between bicyclic ring I of apramycin containing a 6′-OH group and A1408, (c) between ring I containing a 6′-NH_3^+ group and A1408, and (d) between ring I containing a 6′-OH group and G1408 (hydrogen atoms are added for better understanding of hydrogen bonds). (e) Detailed interaction of ring I with the bacterial A-site molecular switch. (f) Detailed interaction of ring II (2-DOS) with the bacterial A-site molecular switch. Hydrogen bonds and a C–H . . . O interaction are shown in dashed lines.

codon. Position 4 is always used for connection to ring I. In the disubstituted aminoglycosides, one of the two positions 5 and 6 is used for connection to ring III and the other is free from any interaction. Naturally, the aminoglycoside-inactivating enzymes, AAC (1) and AAC (3), specifically modify NH_3^+ groups at positions 1 and 3, respectively [40].

19.7 Dual Roles of Extra Rings: Improving the Binding Affinity and Eluding Defense Mechanisms

The simplest 4-monosubstituted aminoglycoside neamine carries only rings I and II and binds to the A site [18] but shows the lowest antibacterial activity (Table 19.1) [6, 7]. These two rings are necessary for the specific binding to the A site, while the extra rings are important both for increasing the binding affinity and for introducing chemical diversity in order to escape bacterial defense mechanisms based on the enzymatic modifications of the antibiotics.

The 4,5-disubstituted aminoglycosides have one to three extra rings; ribostamycin has a ring III, neomycin B and paromomycin have rings III and IV, and lividomycin B has rings III, IV, and V. The pentose ring III uses its O2″ and O5″ atoms for interaction with the $N4_{C1407}$ and $N7_{G1491}$ atoms, respectively (Figure 19.5a). Ring IV, attached to the O3″ atom of ring III, recognizes G1405 through only one hydrogen bond N2‴–H . . . O2P. Ring V, attached to the O4‴ atom of ring IV, uses two OH groups at positions 2⁗ and 3⁗ for interaction to the $O1P_{C1404}$ atom. As both rings IV and V interact with the phosphate oxygen atoms, these recognitions are not sequence specific.

The 4,6-disubstituted aminoglycosides commonly have only one extra ring. Ring III can be grouped into two types (Figure 19.5b). Type A in gentamicin and geneticin has an $NHCH_3$ group at position 3″, a CH_3 group at position 4″, and two OH groups at positions 2″ and 4″. Type B in tobramycin, kanamycin A, and kanamycin B has an NH_3^+ group at position 3″ and three OH groups at positions 2″, 4″, and 6″. Three hydrogen bonds from the O2″ and N3″ atoms to the O6, N7, and O2P atoms of G1405 are commonly observed for both types. The 4″-OH group in type A is in an axial position, points to the A site, and interacts with $O2P_{U1406}$. On the other hand, the 4″-OH group in type B is in an equatorial position and points toward the solvent region, thereby losing a hydrogen bond. The O6″ in type B is not involved in the A-site recognition. In ring III, only one functional group 2″-OH is known to be modified by aminoglycoside-inactivating enzymes ANT(2″) and APH(2″) [40].

A 4-monosubstituted aminoglycoside apramycin, which can also be classified as the aminocyclitol group, has a ring III that recognizes the G1491 residue through O5″ . . . H-$N2_{G1491}$ and O2″ . . . H-$O2'_{G1491}$ (Figure 19.5c). As a consequence, a pseudo-base triple C1409-G1491-ring III is formed. A 6″-OH group interacts with the O4′ atom of A1410, but 3″-OH and 4″-NH_3^+ groups are free from any interaction.

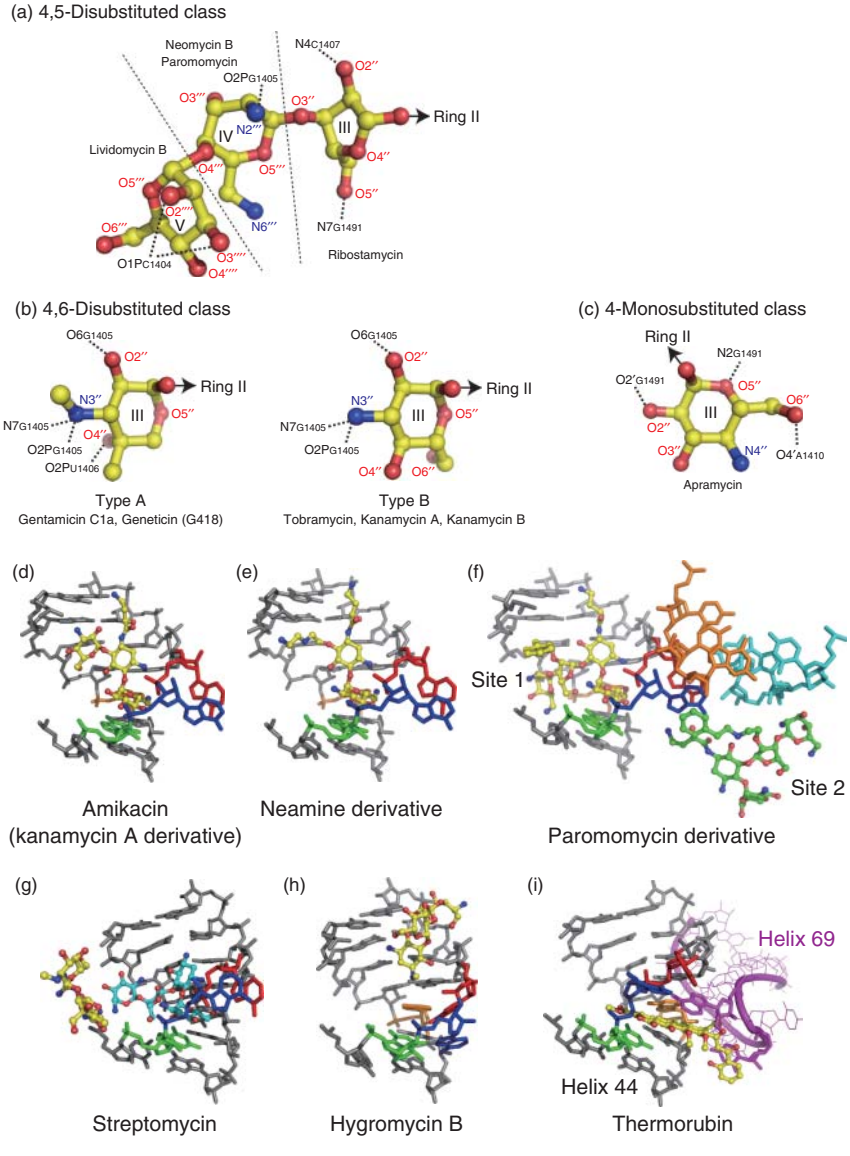


Figure 19.5 (a) Detailed interactions of ring III, IV, and V of 4,5-disubstituted, (b) ring III of 4,6-disubstituted and (c) ring III of 4-monosubstituted aminoglycosides with the bacterial A-site molecular switch. Specific binding of aminoglycosides to the bacterial A-site molecular switch; (d) amikacin (a kanamycin A derivative), (e) a neamine derivative, (f) a paromomycin derivative (yellow and green), (g) streptomycin (yellow), and paromomycin (cyan), (h) hygromycin B, and (i) thermorubin.

19.8
Binding of Semisynthetic Aminoglycosides to the Bacterial A Sites

Several semisynthetic compounds with improved antibiotic activity and resistance to aminoglycoside-modifying enzymes have been designed during recent years [41–43]. The binding modes of some of these compounds to the bacterial A site have been revealed by X-ray analyses.

A semisynthetic aminoglycoside amikacin, which has been in clinical use since 1977, was developed by acylation of kanamycin A with a γ-amino-α-hydroxybutyryl (L-haba) group at position 1 of ring II (2-DOS) [44, 45]. The compound is known to be a poor substrate for various aminoglycoside-modifying enzymes presumably due to steric hindrance caused by the L-haba group [46, 47], and its antibacterial activity is generally equal to or greater than that of the parent compound kanamycin A against various species including aminoglycoside-resistant strains [44, 45]. In the crystal structure of the bacterial A site in complex with amikacin [19], all direct contacts observed for the parent compound kanamycin A were conserved. Two additional contacts are provided by the L-haba group that interacts with the N4 atom of C1496 and the O6 atom of G1497 in the upper side of the A-site helix (Figure 19.5d). Therefore, it can be concluded that the introduction of the L-haba group is an effective mutation for obtaining aminoglycosides with a higher affinity to the A site and less affinity for the several aminoglycoside-modifying enzymes.

Neamine derivatives designed by Mobashery and coworkers [48] have an L-haba group and an amine-containing aliphatic group at positions 1 and 6 of ring II (2-DOS), respectively. These compounds were shown to be highly active against various resistant and pathogenic bacteria. It has been confirmed by X-ray analyses [49, 50] that their binding modes to the bacterial A site are basically the same as that of natural aminoglycosides. The L-haba group makes direct contacts with the upper side of the A site as observed for amikacin. The aliphatic group is structurally flexible and makes electrostatic interactions with the phosphate groups of the A-site helix by using the secondary amine and the terminal amino group (Figure 19.5e).

Hanessian and coworkers [17, 20–23] have designed a series of 2″-*O*-substituted ether analogs of paromomycin, and the detailed interactions with the bacterial A site have been revealed by X-ray analyses. In the A site, rings I and II adopt the conserved orientation and position, but rings III and IV are oriented very differently from the parent compound paromomycin. The ether chain attached at position 2″ of ring III extends across the deep/major groove of the A-site helix and points toward the solvent without any direct contact with RNA atoms. As a result, ring III rotates 40° around the β-D-ribofuranosyl linkage to the paromamine unit, the sugar pucker of ring III changes from C2″-endo to C3″-endo and ring IV rotates 90° compared to paromomycin in the A site (Site 1 in Figure 19.5f). Besides the conserved binding mode to the A site, one of the paromomycin derivatives possessing an L-haba group at position 1 of ring II (2-DOS) and an ether chain with an *O*-phenethylaminoethyl group at position 2″ of ring III displays a new specific binding involving an A-minor motif at the crystal packing interface that mimics interaction between two bulged-out adenines from the A site and the

codon–anticodon stem of the mRNA–tRNA complex (Site 2 in Figure 19.5f) [21]. The compound with the dual modification shows a superior level of antibacterial activity compared to the parent paromomycin and other paromomycin derivatives in both gram-negative and gram-positive bacteria, suggesting that the compound might affect protein synthesis in two different ways: (i) specific binding to the A site maintains the ''on'' state and (ii) a new specific binding to the A-minor motif stabilizes complex formation between ribosome and mRNA–tRNA complex.

19.9
Binding of Aminoglycosides to the Antibiotic-Resistant Bacterial Mutant and Protozoal Cytoplasmic A Sites

It has recently been confirmed by an X-ray analysis that a 4,6-disubstituted compound geneticin binds to the ''on'' state of the bacterial A1408G antibiotic-resistant mutant and protozoal cytoplasmic A sites in the same manner as observed in the bacterial wild-type A site (Figure 19.1d) [30]. In the A sites carrying the G1408 residue and the C1409=G1491 base pair (Figure 19.3), ring I with the 6′-OH group stacks on G1491 and forms a pseudo pair with two hydrogen bonds and one C–H . . . O interaction, O6′ . . . H–$N2_{G1408}$, O5′ . . . H–$N1_{G1408}$ and C1′–H . . . $O6_{G1408}$ (Figure 19.4d). However, a ring I with a 6′-NH_3^+ group cannot form a pseudo pair because the NH_3^+ group repels both the N2–H and N1–H groups of G1408, which constitutes the main explanation for the observation that the A1408G mutation confers high-level antibiotic resistance to the 6′-NH_3^+ subclass but moderate resistance to the 6′-OH subclass such as geneticin, paromomycin, and lividomycin (Table 19.1).

19.10
Binding of Aminoglycosides to the Human A Sites

Although crystal structures of the human mitochondrial and cytoplasmic A sites in complex with the 4,5- and 4,6-disubstituted aminoglycosides have not been solved yet, the toxicity to humans has been considered to originate from the same molecular mechanism owing to the similarity at the secondary structure level of these human A sites to the bacterial and protozoal cytoplasmic A sites (Figure 19.3). On the other hand, the 4-monosubstituted aminoglycosides, apramycin and a synthetic compound NB33, bind both specifically to and stabilize the ''off'' state of the human cytoplasmic A site (Figure 19.1d) [32, 33]. In the cytoplasmic ''off'' state, the two switchable adenines form a *trans* sugar-edge/Hoogsteen A1493-G1408 and a *cis* sugar-edge/Watson–Crick A1492-C1409 base pairs, respectively. The A1491 residue does not form any base pair in the A-site helix and fully bulges out to the solvent. Apramycin and NB33 bind to the deep/major groove of the human cytoplasmic A site and lock the switch ''off,'' but in different binding modes. These aminoglycosides show high affinity to the eukaryotic cytoplasmic A site and inhibit translation in eukaryotic system (Table 19.1) [11, 33].

19.11
Other Aminoglycosides Targeting the A Site but with Different Modes of Action

Streptomycin was the first aminoglycoside antibiotic discovered and isolated by Waksman's group [51]. It is known to interfere with both the initial selection and proofreading steps in protein biosynthesis [52, 53]. It was confirmed by an X-ray study that streptomycin binds to the A site and interacts with the phosphate backbone of helices 44, 27, and 18 (530 loop), and Lys45 from a ribosomal protein S12 (Figure 19.5g) [13]. Helix 27 in the A site has been proposed to switch its conformation between two alternative states: a restrictive state with nucleotides 888–890 paired with 910–912 and a ribosomal ambiguity (*ram*) state with nucleotides 885–887 paired with 910–912 [28, 54]. The A site with the former state has a low aminoacyl-tRNA affinity and the A site with the latter state has a higher affinity. As tightly bound streptomycin preferentially stabilizes the *ram* state in the crystal structure, the drug would affect both initial selection and proofreading of aminoacyl-tRNA.

Hygromycin B is an aminocyclitol antibiotic having a dual ester linkage between two of its three sugar rings, which can also be categorized as the 5-monosubstituted 2-DOS aminoglycoside. The drug inhibits the translocation step in both bacteria and eukaryotes [55]. The binding site of hygromycin B found in a crystal structure overlaps with that of other aminoglycosides [56]. It binds to the upper side of the A-site RNA helix and interacts with universally conserved residues C1403, G1405, G1494, U1495, C1496, and U1498 (Figure 19.5h). As the translocation step accompanies a conformational change of helix 44 to which hygromycin B binds, the drug could inhibit that process specifically.

19.12
Aminoglycosides that Do Not Target the A Site

Aminoglycosides that cause mRNA miscoding are known to inhibit ribosome recycling too [57]. A structural basis for aminoglycoside inhibition of bacterial ribosome recycling has been proposed by an X-ray study of the *Escherichia coli* 70S ribosome [58]. In bacteria, the ribosome recycling factor (RRF) helps separate the 70S ribosome into its small and large subunits. Binding of RRF to the ribosome causes helix 69 of the large subunit to swing away from the subunit interface. The conformational change breaks key bridges between the small and large ribosomal subunits that are crucial for subunit association. Aminoglycosides bind to the deep/major groove of helix 69 and completely restore the contacts between the subunits that are disrupted by RRF, thereby inhibiting ribosome recycling.

Spectinomycin is an aminocyclitol inhibiting EF-G-catalyzed translocation of the peptidyl-tRNA from the A to P sites (peptidyl-tRNA binding site) [59]. Crystal structures show that spectinomycin binds in the minor groove at one end of helix 34, at the neck region of the 30S subunit connecting between the head and platform regions [13, 60]. Translocation accompanies movement of the head region. Spectinomycin binds near this pivot point of the head and sterically blocks the movement.

Kasugamycin is an aminoglycoside that was initially discovered as a drug against a fungus causing the rice blast disease [61] and was later revealed to be active also against several bacterial strains [62]. In an X-ray study [63], two binding sites of kasugamycin were observed within the path of the mRNA; site 1 is located between the P and E sites and site 2 overlaps both the mRNA and tRNA in the E site (the ribosome exit or deacylated-tRNA binding site). The aminoglycoside indirectly inhibits binding of initiator fMet-tRNA to the P site by disturbing the mRNA–tRNA codon–anticodon interaction.

Unlike other aminoglycosides targeting the A site, spectinomycin and kasugamycin both lack the 2-DOS ring necessary for the specificity to the A site.

19.13 Nonaminoglycoside Antibiotic Targeting the A Site

Tetracycline antibiotics have been used against a wide range of both gram-negative and gram-positive bacteria. In recent years, however, their clinical uses have been limited owing to widespread resistant strains. It has been proposed by biochemical studies that they inhibit protein synthesis by blocking the binding of aminoacyl-tRNA to the A site [64, 65]. Crystal structures of the 30S ribosome in complex with tetracycline solved by two different groups confirmed that one of several binding sites of tetracycline is located at the A site but on the opposite side of the tRNA anticodon stem, thereby preventing binding of aminoacyl-tRNA to the A site by direct steric hindrance [56, 66].

Thermorubin is a natural product with antibacterial activity isolated from the thermophilic actinomycete *Thermoactinomyces antibioticus* [67]. Although its structure with a linear tetracyclic core resembles that of tetracycline antibiotics, it binds to the ribosome at a different location [68]. Thermorubin binds to the A-site molecular switch by stacking on the Watson–Crick C1409-G1491 base pair and bridges between helix 44 of the small subunit and helix 69 of the large subunit (Figure 19.5i). Rearrangement of two bases on helix 69 induced by binding of thermorubin may block the binding of aminoacyl-tRNA to the A site, thereby inhibiting the initiation phase of protein synthesis.

19.14 Conclusions

Aminoglycosides have a long history as antibiotic drugs since the discovery and isolation of streptomycin in 1944 [51]. However, several issues still remain to be resolved. The clinical and veterinary use of aminoglycosides has resulted in the rapid spread of antibiotic-resistant strains. The toxicity to mammals is also a critical problem that limits intensive use of aminoglycosides. In addition, the ''old'' antibiotic agents have recently been attracted as ''new'' drugs for the possible treatment of human genetic diseases including cystic fibrosis and Duchenne muscular dystrophy [41, 42]. These genetic diseases are caused by nonsense mutations that introduce premature stop codons and lead to synthesis of nonfunctional shortened proteins. Certain aminoglycosides can bind to and

disturb the function of the human cytoplasmic A site, which induces readthrough of premature stop codons, thereby recovering production of functional full-length proteins. Developing novel aminoglycosides with a targeted activity and less toxicity is the most challenging task. The structural basis for the action of aminoglycosides to the ribosomal A sites are reviewed in this chapter. In the future, the structural basis of recognition and binding to human mitochondrial and cytoplasmic A sites should contribute to that aim.

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20
Peptidyltransferase Inhibitors of the Bacterial Ribosome

Daniel Wilson

20.1
Peptide Bond Formation and Its Inhibition by Antibiotics

The central enzymatic function of the ribosome is peptidyltransferase, that is, formation of peptide bonds, the active site for which is located on the large ribosomal subunit (reviewed by Simonovic and Steitz [1] and Polacek and Mankin [2]) (Figure 20.1a). This ribosomal active site is composed exclusively of rRNA nucleotides (Figure 20.1b,c), indicating that the ribosome is a ribozyme [3]. The peptidyltransferase reaction involves the nucleophilic attack by the α-amino group of the aminoacyl-tRNA bound in the A-site of the ribosome on the carbonyl-carbon of the peptidyl-tRNA located in the P-site (Figure 20.1c,d), which resolves to give a peptidyl-tRNA in the A-site (elongated by one amino acid) and a deacylated (or uncharged) tRNA in the P-site. This enzymatic activity is the target for a large range of diverse compounds with differing specificities across the phylogenetic domains of life. This review focuses on the major classes of bacterial peptidyltransferase inhibitors that have been well characterized biochemically as well as structurally (Table 20.1). Thus, this review focuses on eight distinct classes of antibiotics that bind at the peptidyl transferase center (PTC), the majority of which bind within the A-site of the PTC: specifically, these include the puromycins, chloramphenicols, oxazolidinones, lincomycins, and sparsomycins. In addition, blasticidin S, which binds exclusively at the P-site, and the pleuromutilins and streptogramins A, which overlap with both the A- and P-sites, are also covered. A simple view of the mode of inhibition of these peptidyltransferase inhibitors is that by binding at the PTC they compete with and prevent the binding of the natural substrates, aminoacylated-tRNAs on the ribosome, or at least perturb the correct placement of the CCA-end (CCA-aa) of the A- and/or P-site tRNA. However, the ribosome is dynamic and the substrates (aa-tRNAs) vary dramatically in their chemical properties, suggesting that specific functional states of the ribosome may be targeted by particular classes of antibiotics – aspects that we are only just beginning to understand.

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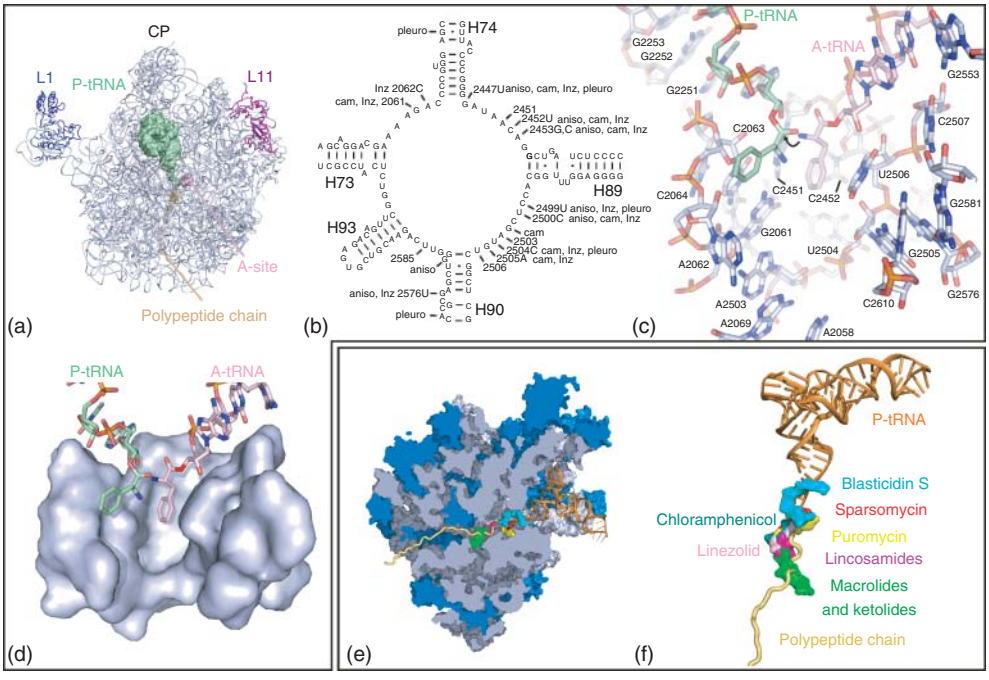

Figure 20.1 The ribosomal peptidyltransferase center as a target for antibiotics. (a) Interface view of the large ribosomal 50S subunit indicating the binding positions of the P-site tRNA (green), the CCA-end of the A-site tRNA (pink), and the path of the nascent polypeptide chain through the exit tunnel (tan). Landmark proteins L1 (blue) and L11 (magenta) as indicated. (b) Secondary structure of the peptidyltransferase ring of the *E. coli* 23S rRNA with the mutation sites in bacteria (blue) and archaea (purple) that confer resistance to peptidyltransferase inhibitors indicated. (c) Ribosomal RNA nucleotides of the peptidyltransferase center that form a pocket for the accurate positioning of the aminoacylated CCA-ends of the A- (pink) and P-tRNA (green). An arrow indicates the nucleophilic attack of the α-amino group of the A-tRNA on the carbonyl carbon of the P-tRNA that results in a peptide bond between the amino acid in the A-site and nascent polypeptide chain in the P-site. (d) A surface representation of (c) showing how the rRNA nucleotides of the ribosomal peptidyltransferase center form a binding pocket for the terminal ends of the A- and P-tRNAs. (e) A transverse section of the large ribosomal subunit (rRNA, gray; ribosomal proteins, blue), with a superimposition of binding sites of peptidyltransferase inhibitors (colored), the P-tRNA (orange), and the path of nascent polypeptide chain (tan). (f) Enlargement of the superimposition of binding sites of peptidyltransferase inhibitors (colored) with the P-tRNA (orange) from (e).

20.2
Puromycin Mimics the CCA-End of tRNAs

Puromycin is a structural analog of an aminoacylated-A76 of tRNA, with the important exception that the amino acid is linked to the ribose sugar via an

Table 20.1 Summary of available structures of antibiotics targeting the PTC.

Antibiotic	Species[a]	Complex	PDB ID[b]	References
Chloramphenicols				
Chloramphenicol	D.r	50S	1K01	[4]
Chloramphenicol	H.m	50S	1NJI	[5]
Chloramphenicol	E.c	70S	3OFA-D	[6]
Chloramphenicol	T.t	70S	3OGE/Y, 3OH5/7	[7]
Lincosamides				
Clindamycin	D.r	50S	1JZX	[4]
Clindamycin	E.c.	70S	3OFX-Z, 3OG0	[6]
Clindamycin	H.m.	50S-G2099A	1YJN	[8]
Nucleotide analogs				
Anisomycin	H.m	50S	1K73	[5]
Anisomycin	H.m	50S	3CC4	[9]
Blasticidin S	H.m	50S	1KC8	[5]
Puromycin[c]	D.r	50S	1NJ0	[10]
Puromycin[c]	H.m	50S	1FG0	[11]
Puromycin[c]	H.m	50S	1FFZ	[11]
Puromycin[c]	H.m	50S	1KQS	[12]
Sparsomycin	D.r	50S	1NJN	[10]
Sparsomycin + ASM	D.r	50S	1NJM	[10]
Sparsomycin + CCA-pcb	H.m	50S	1M90	[5, 13]
Oxazolidinones				
Linezolid + CC-Puro	H.m	50S	3CPW	[14]
Linezolid	D.r	50S	3DLL	[15]
Pleuromutilins				
Retapamulin	D.r	50S	2OGO	[16]
SB-571519	D.r	50S	2OGM	[16]
SB-280080	D.r	50S	2OGN	[16]
Tiamulin	D.r	50S	1XBP	[17]
Tiamulin	H.m	50S	3G4S	[18]
Streptogramins				
Dalfopristin (+ Quinupristin)	D.r	50S	1SM1	[19]
Virginiamycin M + S	H.m	50S	1YIT	[8]
Quinipristin	H.m	50S-G2099A	1YJW	[8]

[a] T.t, E.c, D.r, and H.m correspond to *Thermus thermophilus*, *Escherichia coli*, *Deinococcus radiodurans*, and *Haloarcula marismortui*, respectively.
[b] Protein data bank (pdb) files for each antibiotic complex can be downloaded at *http://www.rcsb.org/pdb/*
[c] (a) Puromycin is in the form of ACC-puromycin; (b) puromycin attached to a 13 bp minihelix and thus mimics a tyrosyl-tRNA acceptor stem; (c) puromycin in the form of an analog of A-site aa-tRNA and P-site peptidyl-tRNA covalently linked by the tetrahedral carbonyl carbon intermediate during peptide bond formation (Yarus inhibitor); and (d) the products of the PTF reaction where the A-site has CCA and the P-site contains puromycin in the form of CC-puromycin-phenylalanine-caproic acid-biotin.

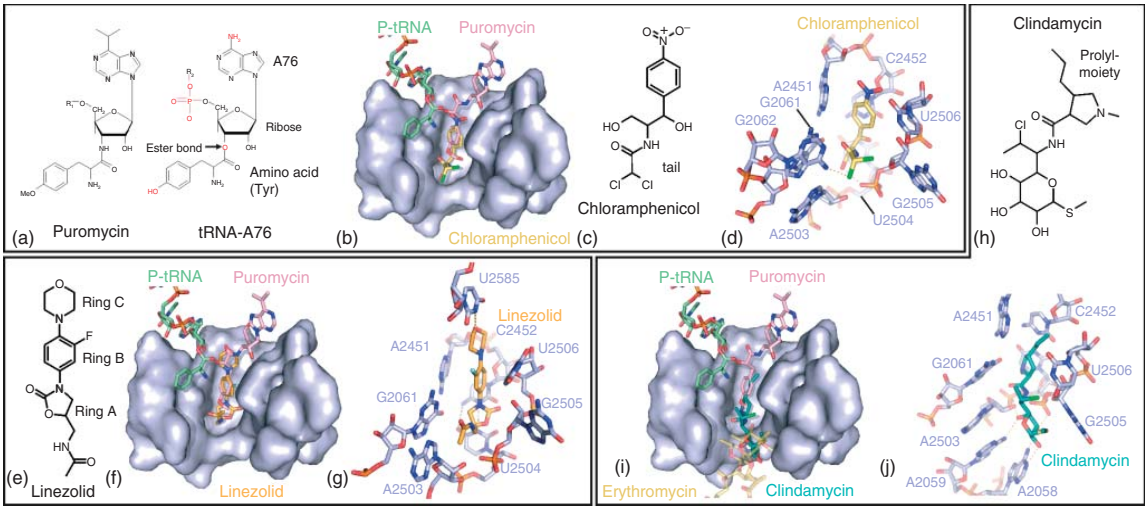

Figure 20.2 Overlapping binding sites of puromycin, chloramphenicol, linezolid, and clindamycin (a) Comparison of the chemical structure of puromycin with the terminal adenine (A76) aminoacylated with tyrosine. Differences between puromycin and the physiological tRNA substrate are indicated in red on tRNA-A76. (b) Relative binding position of puromycin (pink) [5, 13], P-tRNA (green), and chloramphenicol (yellow) at the ribosomal PTC (blue surface). (c) Chemical structure of chloramphenicol. (d) Interaction of chloramphenicol at the *E. coli* PTC [6]. (e) Chemical structure of linezolid. (f) Relative binding position of linezolid (orange) [14, 15, 23], P-tRNA (green), and puromycin (pink) [5, 13] at the ribosomal PTC (blue surface). (g) Interaction of linezolid at the bacterial PTC [15]. (h) Chemical structure of clindamycin. (i) Relative binding position of clindamycin (teal) [6], P-tRNA (green), puromycin (pink) [5, 13], and erythromycin [6] (yellow) at the bacterial PTC (blue surface). (j) Interaction of clindamycin at the *E. coli* PTC [6].

amide rather than ester linkage or bond (Figure 20.2a). Analogous to aa-tRNAs, puromycin binds within the A-site pocket of the PTC (Figure 20.2b) and undergoes peptidyltransfer to accept the nascent polypeptide chain from the P-tRNA, thus covalently linking it to the drug. Subsequently, the peptidyl-puromycin dissociates from the ribosome owing to its low affinity, being bound at the A-site only via the A76, that is, lacking additional interactions available to the entire tRNA molecule. Moreover, should the peptidyl-puromycin rebind at the P-site, translation cannot continue because the amide bridge of puromycin cannot be hydrolyzed by the incoming aa-tRNA. Thus, puromycin effectively terminates peptide-chain elongation by exploiting the ribosomal peptidyltransferase activity. Puromycin is produced by *Streptomyces alboniger* and inhibits growth across all three domains of life. For this reason, the drug is not used clinically, but has been an important tool for studying the peptidyltransferase reaction. In fact, the classical definitions of the ribosomal A- and P-sites are based on the inability or ability, respectively, of aa- or peptidyl-tRNAs to react with puromycin. A series of CC-puromycin analogs mimicking substrates (Figure 20.2b) [13], transition intermediates [11], and post-peptide bond formation products [12] have been visualized bound to the ribosome (Table 20.1), contributing to our atomic understanding of the mechanism of peptide bond formation [20, 21] (reviewed by Simonovic and Steitz [1], Polacek and Mankin [2] and Steitz [22]).

20.3 Chloramphenicols Inhibit A-tRNA Binding in an Amino-Acid-Specific Manner

Chloramphenicol was originally isolated from *Streptomyces venezuele* and contains a *para*-nitrophenyl ring attached to a dichloroacetamido tail (Figure 20.2c). Chloramphenicol displays broad-spectrum activity, inhibiting a wide range of gram-positive and gram-negative bacteria, but does not inhibit translation on eukaryotic 80S ribosomes [24]. Chloramphenicol has been crystallized in complex with three bacterial ribosomal particles, *Deinococcus radiodurans* 50S [4], *Escherichia coli* 70S [6], and *Thermus thermophilus* 70S [7] (Table 20.1), revealing a primary binding site within the A-site pocket of the PTC (Figure 20.2b). The higher resolution structures [6, 7] reveal that the phenyl ring binds analogously and planar to a phenyl moiety of an A-tRNA (Figure 20.2b), consistent with the observation that chloramphenicol interferes with the puromycin reaction, as well as the binding of small tRNA fragments to the A-site of the PTC [25]. On the bacterial ribosome, the phenyl ring of chloramphenicol is sandwiched between A2451 and U2506 where it establishes stacking interactions with C2452 (Figure 20.2d). In addition, the tail of chloramphenicol can form hydrogen-bond interactions with the base of G2061 and the backbone of G2505 (Figure 20.2d). Mutations that confer resistance to chloramphenicol generally map directly within the binding site, for example, 2451, 2503, and 2504, or nearby, for example, 2447 and 2452. Chloramphenicol has also been

crystallized in complex with the archaeal 50S subunit; however, in contrast to bacteria, no density for the drug was observed in the primary binding site [5]. Instead, a secondary binding site was observed deeper within the tunnel, overlapping the binding site of the macrolide and ketolide antibiotics. Curiously, equilibrium dialysis studies have indicated the presence of two binding sites for chloramphenicol on bacterial ribosomes – a high (K_d 2 μM) and low (K_d 200 μM) affinity site [26] that could reflect the primary and secondary binding sites, respectively. Indeed, cross-linking of chloramphenicol to *E. coli* and archaeal *Halobacterium halobium* ribosomes identified modifications within the macrolide binding site, consistent with the secondary chloramphenicol binding site [27]. Nevertheless, this secondary site is unlikely to be critical for the inhibitory mechanism of the drug because most resistance mutations and modifications cluster around the primary binding site within the A-site of the PTC [24, 28]. Indeed, methylation of A2503 by the methyltransferase Cfr is sufficient to confer resistance to chloramphenicol in *E. coli* [28]. Chloramphenicol is well known as an elongation inhibitor because addition of the drug to growing bacterial cells stabilizes polysomes. However, it should be noted that the ability of chloramphenicol to inhibit the peptidyltransferase reaction may be dependent on the nature of the substrates, such that tRNAs bearing bulky aromatic side chains are less prone to inhibition than tRNAs bearing smaller or charged amino acids, such as glycine or lysine [29–31]. Indeed, the *in vitro* synthesis of poly(U)-dependent poly(Phe) is poorly inhibited by chloramphenicol, whereas chloramphenicol inhibits poly(A)-dependent poly(Lys) significantly better. This may suggest that tRNAs bearing the large aromatic residues, such as phenylalanine, are more effective at displacing chloramphenicol from the primary binding site. Chloramphenicols also influence translational accuracy, promoting stop codon suppression and frameshifting, but unlike the aminoglycosides, do not induce misincorporation [32, 33].

20.4 The Oxazolidinones Bind at the A-Site of the PTC

Linezolid is a synthetic compound belonging to the oxazolidinone class of antibiotics and is used clinically to treat a variety of gram-positive infections [34]. Linezolid is composed of three aromatic rings with an acetamidomethyl tail attached to the pharmacokinetic oxazolidinone ring A (Figure 20.2e). A wealth of biochemical and structural evidence [24] indicate that oxazolidinones bind at the PTC, in a position overlapping the aminoacyl-moiety of an A-site tRNA (Figure 20.2f). Indeed, there is an excellent overall agreement in the linezolid-binding position derived from cross-linking data [23] and subsequently visualized position from X-ray crystallography [14, 15] (Table 20.1). In all cases, the morpholino ring (ring C) approaches U2585, the oxazolidinone ring (ring B) stacks on the base-pair formed by U2504, whereas the acetamidomethyl tail of linezolid extends down the tunnel toward A2503 (Figure 20.2g). All of the nucleotides that comprise the linezolid binding

site are universally conserved and most are known to be functionally important for the peptidyltransferase or peptidyl-tRNA hydrolysis activity of the ribosome. Of these eight nucleotides, linezolid resistance mutations have been reported at only three of these positions, namely, C2452U and U2504C in archaea [35] and G2505A in eubacteria [36]. Although the majority of the mutations that confer linezolid resistance involve nucleotides that do not directly interact with the drug, all of the mutation sites are adjacent to at least one of the universally conserved residues comprising the linezolid binding site. With the exception of G2576, which stacks directly onto G2505, all of the remaining mutation sites are clustered around the U2504-C2452 base pair on which the oxazolidinone ring stacks (Figure 20.2g). Despite the detailed structural characterization of the interaction of linezolid at the PTC, the exact mechanism of action of oxazolidinones still remains unclear. On the basis of the binding position, linezolid should prevent correct placement of the aminoacyl moiety of the A-tRNA, analogous to chloramphenicols, and thus inhibit peptide bond formation. Surprisingly, there are conflicting reports as to the ability of different oxazolidinone members to inhibit the puromycin reaction [24, 37]. Moreover, when inhibition is reported, nonphysiologically high concentrations of the drug (~1 mM) are required. Similarly, while oxazolidinones are observed to compete effectively for ribosome binding with chloramphenicols, lincosamides, and puromycins, the IC_{50}s are in the ~1 mM range [38, 39]. In contrast, the IC_{50} for linezolid determined using *in vitro* translation systems is significantly lower (~1–10 μM) [39–41], suggesting that linezolid targets a particular functional state of the ribosome. In this respect, it is interesting that the quality of the electron density for linezolid was improved by the presence of an aminoacyl-tRNA mimic CCA-Phe [14], suggesting that the interaction of linezolid with the P-tRNA increases the affinity of the drug to the ribosome. Indeed, oxazolidinones have been reported to cross-link to small tRNA-sized molecules on the ribosome; however, the identity of the tRNA was not ascertained [42]. Cross-links were also detected to a ribosomal translation elongation factor LepA (EF-4) [42], suggesting that oxazolidinones target an elongating ribosome. Although the exact function of LepA is unclear, this highly conserved factor can backtranslocate POST ribosomes [43, 44] and stabilize an intermediate PRE state [45, 46]. Although LepA is not essential, it is necessary for optimal translation under a variety of stress conditions [43, 47, 48]. Collectively, these findings suggest that LepA may recognize and bind to the functional state induced by the oxazolidinone, namely, with a partially accommodated A-tRNA [15]. However, it is unclear whether this functional state occurs directly after initiation or during translation elongation. The perturbation of A-tRNA could also explain the loss of translational fidelity observed in the presence of oxazolidinones [32]. Notably, a Δ*lepA* strain has an identical minimal inhibitory concentration (MIC) for oxazolidinones as the wild-type strain [42], indicating that the binding of LepA is a consequence, rather than a cause, of the functional state induced by the oxazolidinones. Future studies should address which functional state of the ribosome the oxazolidinones target and whether A-tRNA accommodation is directly affected.

20.5
Lincosamide Action at the A-Site of the PTC

Two well-characterized lincosamides are the parent compound lincomycin, which is naturally produced by several species of actinomycetes, and clindamycin (Figure 20.2h), a semisynthetic derivative of lincomycin. Clindamycin is active against most gram-positive bacteria as well as some protozoa, such as *Plasmodium falciparum* [49]. Although they exhibit similar affinities for the ribosome, clindamycin is generally a more effective inhibitor and is used clinically, for example, as part of the combination therapy with pyrimethamine and folinic acid for treatment against toxoplasmosis [37]. Analogous to puromycin, chlorampenicol, and linezolid, the lincosamide classes of antibiotics interfere with binding of ribosomal ligands at the A-site, as evident from the inhibition on fMet or AcPhe transfer to puromycin, and by preventing the binding of small tRNA 3′-end mimics, namely, CACCA-Leu to the A-site [50]. Furthermore, lincoasmides compete for ribosome binding with both erythromycin and chloramphenicol [51]. Consistently, crystal structures of clindamycin bound to bacterial [4, 6] and archaeal [8] ribosomes (Table 20.1) locate the drug to the A-site of the PTC (Figure 20.2i). The pyrrolidinyl moiety overlaps the binding site of chloramphenicol as well as the aminoacyl moiety of an A-tRNA, whereas the galactose sugar of clindamycin encroaches on the binding position of the desosamine sugar of the macrolide erythromycin (Figure 20.2i). This latter point is consistent with the observation that a number of strains exhibiting resistance to macrolides also exhibit cross-resistance with lincomycin and streptogramin Bs (the so-called macrolides, lincosamides, and streptogramin B (MLS_B) resistance [52]). In the clindamycin binding site, the majority of the interactions involve hydrogen bonds from hydroxyl groups on the galactose moiety with nucleotides A2503, A2058, and A2059 (Figure 20.2j), providing an explanation as to how modification or mutation of these nucleotides can confer resistance to lincosamides [28, 53]. At the other end of the molecule, the 8′ carbon and prolyl moiety of clindamycin overlap in position with the aminoacyl moiety of A-tRNA (Figure 20.2i), in line with the A-site nature of clindamycin inhibition. It should be noted that some influence of lincosamides on binding of tRNA fragments to the P-site have also been reported [54], which is unexpected based on the crystal structures and warrants further investigation.

20.6
Blasticidin S Mimics the CCA-End of the P-tRNA at the PTC

Blasticidin S, similar to puromycin, is a nucleoside analog; however, unlike puromycin, which mimics the aminoacylated-terminal adenosine (A76) of tRNA, blasticidin S resembles the preceding cytosines, C74 and C75 of tRNA (Figure 20.3a). Blasticidin was discovered as a metabolite of *Streptomyces griseochromogenes* [55] and, similar to sparsomycin, displays activity against all three domains of life [56], being reported to inhibit the peptidyltransferase reaction of both bacterial 70S and eukaryotic 80S ribosomes [57, 58]. Indeed, blasticidin S

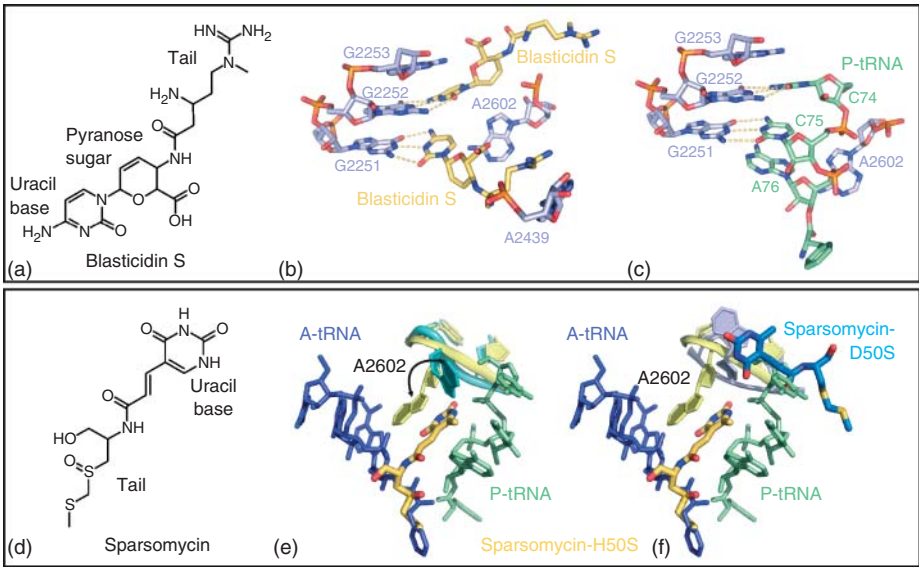

Figure 20.3 Distinct binding modes for blasticidin S and sparsomycin at the PTC. (a) Chemical structure of blasticidin S. (b,c) Interaction of (b) blasticidin S [5] compared with (c) CCA-end of P-tRNA [5, 13], at the PTC. (d) Chemical structure of sparsomycin. (e) Binding site of sparsomycin (orange) bound to archaea (H50S) PTC [5] (yellow), relative to A- (blue), and P-tRNA (green).[13]. A2602 adopts a different conformation (arrowed) when sparsomycin is bound (yellow) compared to PRE-state (cyan). (f) As in (e), but including the sparsomycin (blue) position and A2602 (light blue) from the bacterial *D. radiodurans* (D50S) structure [10].

was first introduced into agriculture as a microbial fungicide to control rice blast caused by *Pyricularia oryzae* [55]. The structure of blasticidin S bound to *Haloarcula marismortui* 50S subunit [5] (Table 20.1) reveals that two molecules bind to the ribosome and interact with nucleotides G2251 and G2252 of the P-loop at the PTC (Figure 20.3b) in a manner analogous to the C74 and C75 of a P-tRNA (Figure 20.3c) [5]. Specifically, the cytosine nucleobase of blasticidin forms Watson–Crick base pairing with the P-loop nucleotides, whereas the *N*-methylguanidine tail of one blasticidin S molecule interacts with nucleotide A2439 (Figure 20.3b) and U2438 (not shown). Consistently, blasticidin S protects A2439 from chemical attack [56] and removal or alteration of the tail of blasticidin S dramatically decreases the inhibitory activity of the drug on translation [59]. Moreover, mutation of U2438C confers resistance to blasticidin S in the archaeon *H. halobium* [60]. Recent evidence suggests that blasticidin S binds transiently at the A-site of the PTC and is then slowly accommodated at the final stable binding on the P-site [61]. Therefore, blasticidin S could act as both a translation initiation inhibitor preventing placement of the initiator tRNA at the P-site of the PTC during subunit joining, but also bind to the P-site of a post-peptide bond formation pretranslocation hybrid-state ribosome when the peptidyl-tRNA is at the A-site and deacylated tRNA is in a hybrid P/E state, and therefore act as an elongation inhibitor.

20.7
Sparsomycin Prevents A-Site and Stimulates P-Site tRNA Binding

Sparsomycin is a nucleoside analog of uracil (Figure 20.3d) produced by *Streptomyces sparsogenes*, which is a potent inhibitor of peptidyltransferase reaction in all organisms studied, both prokaryotic and eukaryotic [51, 62]. Sparsomycin blocks binding of tRNA (and CCA-end fragments) to the A-site of the PTC, while enhancing the affinity of peptidyl-tRNAs (especially N-acetylated aa-tRNAs and CCA-aa fragments) for the P-site. Consistently, sparsomycin resistance results from mutations within the PTC: in the archaeon, *H. halobium* mutation of C2518U and, to a lesser extent, C2471 and U2519 (C2499, C2452, and U2500 in *E. coli*, respectively) confers resistance to sparsomycin [63], as does loss of methylation of U2603 in the archaeon *Halobacterium salinarium* (U2584 in *E. coli*) [64]. Moreover, sparsomycin has been cross-linked to residue A2602 [65] and shown to compete for binding with chloramphenicol and lincomycin [66]. This is in agreement with the structure of sparsomycin bound to the 50S subunit of the archaeon *H. marismortui* (H50S) [5, 13], where sparsomycin stacks between A2602 and the CCA-Phe P-tRNA analog (Figure 20.3e). Binding of CCA-Phe analogs normally distributes evenly between the A- and P-sites in the H50S crystals, but in the presence of sparsomycin, CCA-Phe occupies exclusively the P-site [5, 20]. This is understandable as the conjugated tail of sparsomycin overlaps with the aminoacyl moiety of an A-tRNA (Figure 20.3e) and thus would prevent binding not only of A-tRNA but also chloramphenicol and lincomycin (Figure 20.2b,f), in agreement with previous reports [30, 62]. Surprisingly, the binding position of sparsomycin bound to the bacterial *D. radiodurans* 50S subunit (D50S) is very different (Figure 20.3f). In contrast to the archaeal binding site, in the D50S structure, sparsomycin spans across the P-site and does not encroach on the A-site (Figure 20.3f) [10]. This binding position is hard to reconcile with the ability of sparsomycin to stabilize the P-site substrate, as the reported binding position sterically clashes with P-tRNA (Figure 20.3f). Unlike the H50S crystals, binding of the tRNA substrates in the D50S crystals appears to favor the A-site [10], which may have precluded the formation of the stable functional state observed in the H50S structure. Pretranslocation (PRE) state ribosomes with tRNAs in both A- and P-sites are not protected from the action of sparsomycin, as the drug can induce translocation of the tRNAs such that the peptidyl-tRNA is stabilized at the P-site and the A-site is blocked by the drug [67]. A2602 is at the center of the rotational symmetry of the PTC, where it has been proposed to play a role in guiding the CCA-end of the A-tRNA to P-site during translocation [68], Sparsomycin appears to induce a rotation of A2602 compared to the PRE state conformation (Figure 20.3e,f), which may reflect the two-step reaction mechanism of sparsomycin – a slow initial step that isomerizes slowly to adopt a more stable conformation [69]. In this regard, the position of sparsomycin observed in the D50S structure (Figure 20.3f) may reflect an initial binding event, post-peptide bond formation, where the deacylated tRNA is driven by sparsomycin from the P- to the E-site. Subsequently, the peptidyl-tRNA can then move into the P-site where it is stabilized by sparsomycin, as observed in the H50S structures (Figure 20.3e) [5, 20].

20.8
Pleuromutilins Overlap A- and P-Sites at the PTC

Pleuromutilin was discovered in the 1950s as a natural product of two basidiomycete species, *Pleurotus mutilis* and *Pleurotus passeckerianus* [70]. Modification of the C14 tail of pleuromutilin led to the development of semisynthetic derivatives, such as tiamulin (Figure 20.4a,b) [71], which is used almost exclusively in veterinary medicine. In contrast, retapamulin was approved for human use as a topical agent (Altabax®/Altargo®) to treat skin infections [72]. Pleuromutilins specifically affect eubacteria, displaying excellent activity against *staphylococcal*, *streptococcal*, and anaerobic gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA). Pleauromutilins, such as tiamulin, bind at the PTC in a position overlapping both A- and P-tRNA (Figure 20.4a; Table 20.1) [16–18], puromycin and chloramphenicol (Figure 20.2b), consistent with the finding that tiamulin competes with chloramphenicol, puromycin, and A-tRNAs for ribosome binding [73, 74]. The tricyclic mutilin core (Figure 20.4b), common to all pleuromutilins, inserts into the A-site pocket formed by A2451, A2452, U2504, and G2505, whereas the sulfanyl-acetate substituted C14 tail, seen in tiamulin and retapamulin, can form hydrogen-bond interactions with the base of G2061 (Figure 20.4c) [16, 17]. Variation between the C14 tail influences the mode of interaction formed with G2061 and U2585, and is likely to play an important role in determining the activity profiles of the different pleuromutilin derivatives [75]. Consistently, nucleotides such as G2505 and U2506 have similar chemical modification patterns in the presence of different pleuromutilin derivatives, whereas varying effects are observed for U2584 and U2585 [76]. Resistance to pleuromutilins results from mutations at positions 2055, 2447, 2504, 2572, and 2576 of the 23S rRNA [77–79], many of which give rise to cross-resistance to other antibiotics that bind at the A-site, such as linezolid, chloramphenicol, and clindamycin [80]. Resistance is also associated with alterations in ribosomal protein L3 [77, 81], which is likely to result from indirect perturbation of the binding site. One exception is mutation of U2504 that comprises the pleuromutilin binding site, which is observed in different positions in bacterial and archaeal ribosomes and is thus likely to play an important role for the kingdom specificity of pleuromutilins [18, 80]. It remains unclear exactly which step of translation is targeted by the pleuromutilins. Pleuromutilins have been shown to inhibit the peptidyltransferase reaction and prevent binding of aa-tRNAs to the A-site [73], yet they do not appear to inhibit translation elongation [82]. Instead, pleuromutilins have been proposed to target translation initiation, based on the observations that (i) pleuromutilins destabilize fMet-tRNA binding during initiation complex formation and (ii) the addition of pleuromutilins to intact cells causes the loss of polysomes while stabilizing 70S monosomes [82]. The overlap of pleuromutilins with the P-site would support the inability to correctly place the initiator-tRNA at the P-site during subunit joining.

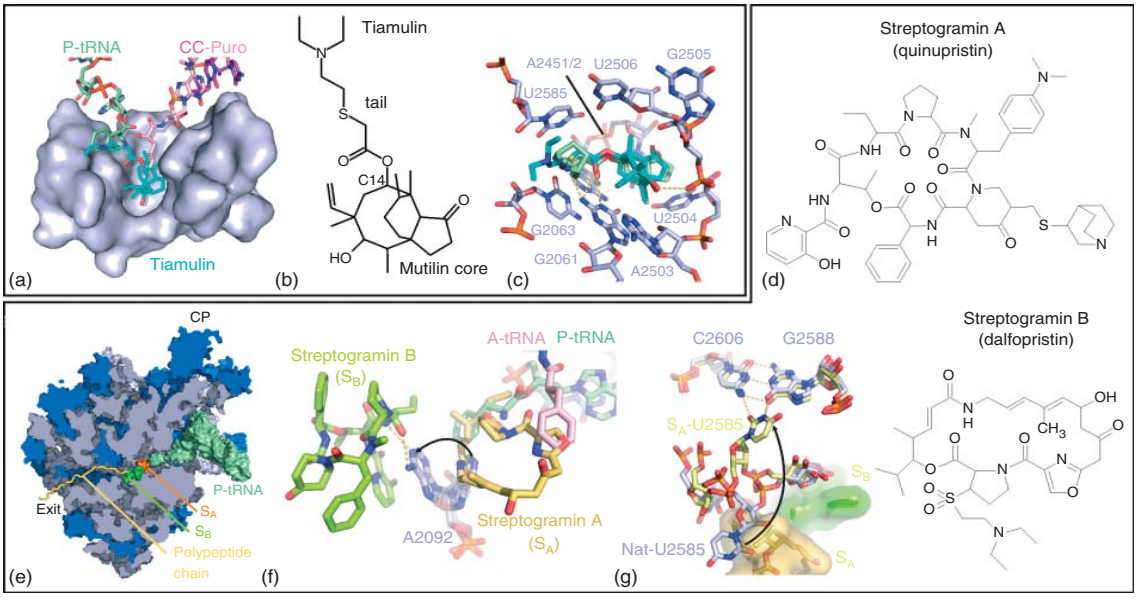

Figure 20.4 Pleuromutilins and streptogramins overlap A- and P-tRNAs at PTC. (a) Relative binding position of CC-puromycin (pink) [5, 13], P-tRNA (green), and tiamulin (cyan) [17] at the ribosomal PTC (blue surface). (b) Chemical structure of tiamulin. (c) Comparison of the interaction of tiamulin (cyan) and retapamulin (green) at the bacterial PTC [16, 17]. (d) Chemical structures of streptogramin A quinupristin and streptogramin B dalfopristin. (e) Transverse section of the large subunit to reveal the binding sites of the streptogramin A (SA, orange), and B (SB, green) antibiotics within the exit tunnel [19]. (f) Binding of streptogramins A (yellow) leads to a shift (as arrowed) in nucleotide A2062 (blue), which stabilizes the binding of streptogramin B (green). Streptogramins A overlap the binding position of both A- (pink) and P-tRNA (pale green) [19]. (g) In bacteria, streptogramin binding leads to a repositioning (as arrowed) of nucleotide U2585, such that it can hydrogen bond (dashed lines) with C2606 and G2588 [19].

20.9
The Synergistic Action of Streptogramins at the PTC

Streptogramins are produced by a variety of *Streptomyces* sp. as a mixture (ratio of 7 : 3) of two chemically unrelated compounds (Figure 20.4d), type A (S_A) and B (S_B). The streptogramins bind to adjacent sites in the 50S subunit, spanning the PTC and ribosomal tunnel (Figure 20.4e; Table 20.1) [5, 8, 19], and inhibit growth of gram-positive bacteria (reviewed by Cocito *et al.* [83] and Mukhtar and Wright [84]). The streptogramin combination of dalfopristin (S_A) and quinupristin (S_B) (Figure 20.4d) is now marketed as Synercid® (Aventis) [85] to treat skin infections, and is also active against some gram-negative and anaerobic bacteria. S_A compounds are cyclic polyunsaturated macrolactones that interfere with binding of tRNA substrates to both A- and P-sites [83, 86, 87], consistent with the overlap in binding position of S_A with the aminoacyl moieties of both A- and P-tRNAs (Figure 20.4f). Suppression of bacterial growth persists for a prolonged period subsequent to the removal of the S_A drug [88, 89], suggesting that S_A binding induces a conformational change within the PTC that is *slowly* reversible. Indeed, S_A alters the reactivity of multiple residues within the PTC [90, 91]: binding of the dalfopristin (S_A) to bacterial large 50S subunit induces a rotational shift in the position of U2585 (compared to the native 50S), promoting interaction with C2606 and G2588 (Figure 20.4g). U2585 plays an important role in the positioning of tRNA substrates and peptide bond formation [20, 21] and therefore the stabilization of U2585 in a nonproductive conformation may contribute to the postantibiotic inhibitory effect of S_A compounds [19]. In contrast, S_B compounds prevent prolongation of the nascent polypeptide chain and induce drop-off of short oligopeptidyl-tRNAs, analogous to the macrolides [92, 93]. The S_B competes with macrolides for ribosome binding [94, 95], and A2058/A2059 mutations confer cross-resistance to MLS_B antibiotics [52, 83]. Moreover, the overlap in the S_B and macrolide binding sites is consistent with the overlap in protection of nucleotides, such as A2058 and A2059, from chemical footprinting [96, 97].

A unique feature of the streptogramins is that they act synergistically *in vivo* and *in vitro*, such that presence of the S_A enhances the binding of the corresponding S_B compound [98]. The synergistic action significantly lowers the concentrations needed to obtain the same level of inhibition when each compound is used separately [99]. Moreover, the synergistic action enables the streptogramins to overcome some resistance mutations [90, 100] and in some cases can convert a bacteriostatic effect into bactericidal lethality. The basis for the synergy between S_A and S_B combinations is most likely related to a rotation of A2062 seen on binding of S_A compounds to the PTC (arrow in Figure 20.4f) [8, 19]. In the new position, A2062 can stabilize the binding of S_B compounds via hydrogen-bond interactions (Figure 20.4f). Consistently, mutations of A2062 result in streptogramin resistance [101]. In summary, the synergistic action of streptogramins blocks both A- and P-sites, and therefore, streptogramins are likely to act during initiation or by inducing peptidyl-tRNA drop-off during an early elongation step.

20.10 Future Perspectives

High-resolution structures of ribosomes alone and in complex with antibiotics have revolutionized our understanding of ribosome structure and function, and enabled us to reinterpret the rich wealth of data relating to the action of antibiotics during translation. Although structures already exist for all major classes of antibiotics that target the ribosome, the search for more potent antibiotics continues to be the challenge for the future in order to overcome the threat of multidrug-resistant bacteria. Despite its conservation, the peptidyltransferase center of the bacterial ribosome appears to remain an excellent target for structure-based drug design. Indeed, the many existing structures of different members of each class as well as the same members bound to ribosomes from different origins [24, 102] have strengthened our understanding of the conserved features that antibiotics utilize to interact with the ribosome, besides highlighting the slight differences that arise due to species-specific differences and/or the functional state of the ribosome – aspects that are critical for the binding and inhibitory activity of the antibiotics.

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21
Antibiotics Inhibiting the Translocation Step of Protein Elongation on the Ribosome

Frank Peske and Wolfgang Wintermeyer

21.1
Introduction

Antibiotics interfere with all steps of protein synthesis, including initiation, elongation, termination, and ribosome recycling. The typical inhibition mechanism is that antibiotics bind to the ribosome, generally to the ribosomal RNA, or, less often, to a translational factor, and inhibit conformational changes that are essential for a particular reaction to take place. The inhibition by antibiotics of the translocation step of elongation provides a number of examples for this type of inhibition. Studying the mechanisms of inhibition has provided a wealth of information about the molecular mechanism of the reaction, in particular about the importance of the structural dynamics of the ribosome.

Many of the known antibiotics are specific to components of the bacterial protein synthesis system and can be used against bacterial infections. A few antibiotics that inhibit eukaryotic protein synthesis are known and are in clinical use against, for instance, fungal infections. This chapter provides examples of the most important antibiotics of the two categories. Antibiotics inhibiting the disassembly of the ribosome into subunits following the termination of protein elongation (''ribosome recycling'') in bacteria are also discussed briefly.

21.2
Translocation: Overview

Following peptide bond formation, the ribosome is present in the pre-translocation state (PRE), which carries peptidyl-tRNA in the A site and deacylated tRNA in the P site. Translocation brings the ribosome into the post-translocation state (POST) in which peptidyl-tRNA occupies the P site and the A site exposes the next codon of the mRNA to accept another aminoacyl-tRNA in the next round of the elongation cycle. Entailing large-scale macromolecular movements and motions within the ribosome, translocation is arguably the most complex step of the elongation cycle. The reaction is driven, both kinetically and thermodynamically, by GTP hydrolysis

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on elongation factor G (EF-G) in bacteria or the homologous eEF2 in eukaryotes. EF-G and eEF2 are large, five-domain GTPases that change conformation upon GTP hydrolysis and release of inorganic phosphate (Pi) and couple these changes to conformational changes of the ribosome that facilitate tRNA–mRNA movement and shift the system toward POST.

Translocation proceeds in steps (Figure 21.1). One, presumably the first, is the formation of the ''hybrid/rotated state'' of the ribosome in the pre-translocation state (PRE), in which the tRNAs assume hybrid binding positions and the subunits of the ribosome undergo a rotation relative to one another. During hybrid-state formation, the deacylated 3′ end of the P-site tRNA moves toward or into the 50S E site and the peptidyl end of the A-site tRNA toward the P site, while the anticodon arms remain fixed to their positions in the 30S P and A sites, respectively [1]. Hybrid-state formation is important for translocation to take place rapidly, as modifying the P-site tRNA of the PRE complex such that its binding to the E site is impaired, that is, the P/E hybrid state cannot form, slows down translocation considerably [2]. In keeping with these results, smaller anticodon stem–loop constructs are not translocated from the P site [3]. Likewise, aminoacyl-tRNA in the A site of a PRE complex, which – unlike peptidyl-tRNA – cannot enter the A/P hybrid state, is translocated very slowly [4]. The same conclusion was reached by studying the effect of ribosome mutations that favored hybrid states by weakening tRNA interactions in the 50S P or A sites [5].

Hybrid-state formation is correlated with a rotation of the 30S subunit relative to the 50S subunit and a swiveling motion of the head of the 30S subunit [6], as shown by cryo-electron microscopy (cryo-EM) [7, 8]. Although the tRNAs can assume their hybrid-state positions spontaneously, or fluctuate between hybrid and classic states, as revealed by single-molecule fluorescence resonance energy transfer (FRET) studies [9, 10], the binding of EF-G-GTP to the PRE complex suppresses the fluctuations and promotes the formation of the hybrid/rotated PRE state

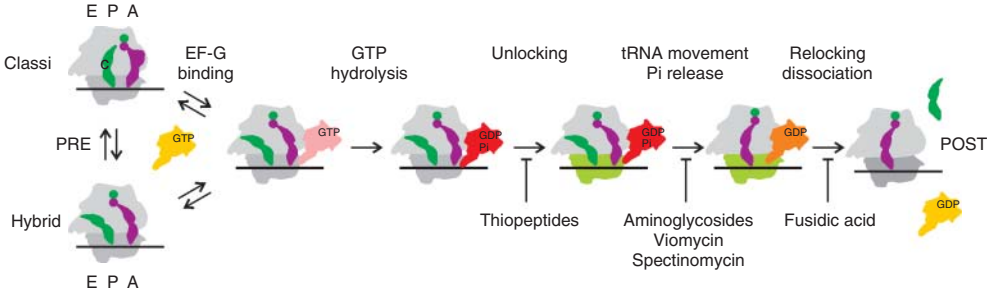

Figure 21.1 Kinetic scheme of translocation. In the pre-translocation state (PRE), the deacylated tRNA (green) fluctuates between the classic P/P and the hybrid P/E state, the peptidyl-tRNA (purple) between A/A and A/P states. The rotation of the subunits is not indicated. The unlocked state of the 30S subunit is indicated by light green coloring. EF-G is depicted in different colors to indicate the different conformations the factor assumes during its functional cycle. The inhibition of specific steps of translocation by antibiotics is indicated.

[11]. The latter state is an essential intermediate of translocation, as blocking the rotation of subunits relative to each other abrogates translocation [12]. Subsequent steps include (i) the tightening of GTP binding to EF-G [13], presumably by closing the nucleotide-binding pocket and/or establishing additional interactions between EF-G and GTP; (ii) GTPase activation, which may result from the same rearrangements; and (iii) GTP hydrolysis. These steps are rapid, GTP hydrolysis taking place at a rate of around $100\,s^{-1}$, independent of the occupancy of the ribosomal tRNA binding sites [14].

EF-G-GTP binding and GTP hydrolysis strongly promote a rearrangement of the ribosome (''unlocking'') that is rate limiting for the following steps of tRNA–mRNA movement and release of Pi [15]. tRNA movement and Pi release are independent of each other, as one step can be inhibited without affecting the other [15–17]. Presumably, unlocking pertains to the 30S subunit mainly and results in an increased mobility of the 30S head domain. That the internal mobility of the 30S subunit is rate limiting for translocation is consistent with the observation that colicin E3 cleavage of 16S rRNA in the decoding site accelerates translocation [18]. The importance of movements within the 30S subunit is also suggested by the inhibition of translocation by antibiotics binding to various regions of the 30S subunit, including the decoding site, the head domain, and the body. These intra-30S movements, however, take place after unlocking, as none of the antibiotics that inhibit tRNA movement affects the rate of Pi release [19]. The conformation of EF-G has to rearrange for these changes to take place, as restricting the conformational flexibility of EF-G by introducing a cross-link between domains 1 and 5 strongly interferes with tRNA movement, but not with GTP hydrolysis and Pi release [20]. The conformational changes seem to involve a region of the molecule centered around the fusidic acid binding site [21].

The movement of the tRNAs bound to the mRNA presumably takes place by diffusion [15], driven by Brownian motion of the tRNAs and/or parts of the ribosome [22]. The movement can take place spontaneously, without EF-G, in both forward and backward directions, depending on the thermodynamic gradient between PRE and POST states [23, 24]. In the presence of EF-G and GTP, the system will always be driven to the POST state, regardless of the thermodynamic gradient of tRNA binding in PRE and POST. In the POST state, EF-G is positioned such that domain 4 reaches into the 30S A site and contacts both mRNA and P-site tRNA [25], thereby preventing the backward movement of the tRNAs. Thus, EF-G assumes the role of the pawl in a Brownian machine that, by preventing backward movement, biases the system into the forward direction. The energy required for the pawl function is provided by GTP hydrolysis. In the PRE state, EF-G is positioned differently, with domain 4 contacting the 30S subunit in the region of the shoulder [26], a position of EF-G that may be instrumental in performing unlocking. The concomitant movement of the tRNAs and EF-G into their post-translocation positions is followed by another rearrangement, relocking, in which the ribosome returns to the locked state. It is not clear whether this rearrangement requires prior Pi release from EF-G. However, the following rearrangement, which enables EF-G to assume a conformation with low affinity for the ribosome, can

take place only after the release of Pi [27]. Subsequently, EF-G dissociates from the ribosome to complete translocation. Inhibiting the conformational change of EF-G by cross-linking [20] or fusidic acid binding (see subsequent text) prevents the dissociation of the factor.

In conclusion, translocation is accelerated and driven in one direction by a series of conformational changes of EF-G, which are induced by ribosome binding, GTP hydrolysis, and the release of Pi. The highly dynamic fluctuation of the ribosome in both EF-G-dependent classical-to-hybrid transition and translocation was also revealed by molecular dynamics simulations [28]. More structural and mechanistic details on translocation can be found in several recent reviews [29–32]. A comprehensive recent review on antibiotics targeting translation in bacteria, including the translocation step, is also available [33].

21.3 Antibiotics Inhibiting Translocation

As outlined earlier, translocation takes place in steps. First, the 3′ ends of the two tRNAs move on the 50S subunit from hybrid states into their POST positions. Subsequently, the anticodon regions of the tRNAs move on the 30S subunit. Movement on both 50S and 30S subunits can be affected by antibiotics. In the following, representative examples for these antibiotics are discussed, beginning with antibiotics targeting various sites in the 30S subunit. Subsequently, the few antibiotics that bind to the 50S subunit and inhibit translocation as well as an antibiotic binding to EF-G are described. Examples of antibiotics that inhibit translocation in eukaryotic, in particular fungal, systems are also included. The chapter concludes with the description of antibiotics that inhibit the function of EF-G in ribosome recycling.

21.3.1 Target: 30S Subunit, Decoding Site

Paromomycin, as a number of other aminoglycoside antibiotics, such as neomycin and gentamicin, binds to the major groove of helix 44 (h44) of 16S rRNA in the 30S decoding center (Figure 21.2) [34]. Ring I of paromomycin inserts into h44, stacking against G1491, and helps to flip out the bases of A1492 and A1493. The flipped-out adenines can form A-minor interactions with anticodon–codon duplexes in the A site [35], thereby stabilizing the binding of the tRNA. This has two effects: one is that the frequency of amino acid misincorporation during decoding is increased, as the conformational change of h44 accelerates GTP hydrolysis of near-cognate ternary complexes, resulting in enhanced A-site accommodation and participation in peptide bond formation of near-cognate aminoacyl-tRNAs [36]. The other effect of paromomycin is a strong inhibition of translocation. The extent of inhibition is closely correlated with the stabilization of A-site binding (both roughly 200-fold), suggesting that the retardation of translocation caused by paromomycin (and related

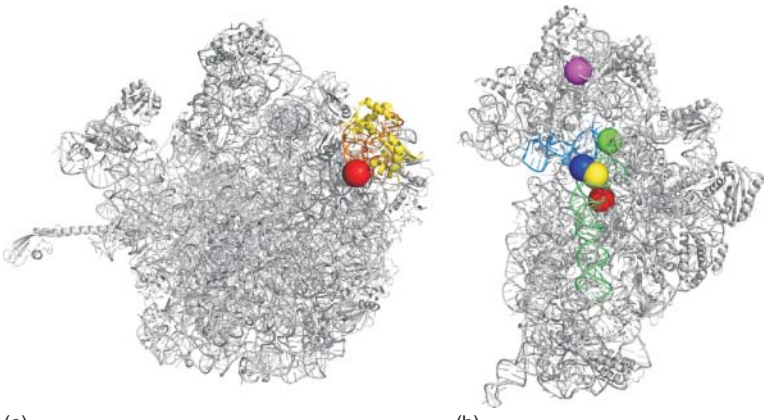

Figure 21.2 Binding sites of antibiotics on (a) 50S and (b) 30S subunits of the bacterial ribosome. The 50S subunit is shown with thiostrepton (red) bound between H43/H44 of 23S rRNA (orange) and the NTD of protein L11 (yellow). Antibiotics bound to the 30S subunit are colored as follows: spectinomycin, green; paromomycin, blue; hygromycin B, yellow; streptomycin, red; GE82832, magenta. Helices h44 and h34 of 16S rRNA are colored green and blue, respectively.

aminoglycosides) is a consequence of increasing the activation energy by stabilizing the PRE state [19]. The effect of the antibiotics is strong enough to preferentially stabilize the PRE state even in the presence of EF-G and GTP [23], a condition which in the absence of antibiotic enforces the formation of the POST state, regardless of the thermodynamic preferences intrinsic to the respective tRNAs.

According to a single-molecule FRET study, the stabilization of the PRE state by aminoglycosides is correlated with a stabilization of the classical/nonrotated conformation of the pre-translocation ribosome [10]. By comparing different aminoglycosides, a quantitative correlation between the extent of stabilization of the classical/nonrotated conformation and the inhibition of translocation was observed, establishing the hybrid/rotated state as a *bona fide* translocation intermediate.

A second binding site of paromomycin has been identified at helix 69 (H69) of 23S rRNA on the 50S subunit and, as discussed in subsequent text, has been implicated in the inhibition by paromomycin of ribosome recycling by ribosome recycling factor (RRF) and EF-G [37].

Hygromycin B, another aminoglycoside antibiotic, binds to h44 of 16S rRNA right above the decoding site and the binding site of paromomycin (Figure 21.2), making contacts to nucleotides in the regions 1490–1500 and 1400–1410 [38, 39]. Hygromycin B inhibits translocation about 300-fold, while it stabilizes peptidyl-tRNA binding in the A site only 30-fold [19]. This indicates that the inhibition of translocation by hygromycin B is due in part to a stabilization of the PRE state, resembling the effect of other aminoglycosides. The other part may be due either to the inhibition of a conformational change in h44 that is required for translocation or to the induction of a conformation that is detrimental for translocation. Hygromycin B induces a particular conformation around its binding

site, which differs from that induced by, for example, paromomycin. In particular, the critical residue A1492 in the complex with hygromycin B remains in its original position within h44, whereas residue A1493 assumes a position between the tRNA anticodon loops in A and P sites where it can interact with the A-site codon [39]. It is, therefore, possible that A1493 in the position induced by hygromycin B forms a steric block that hinders tRNA movement on the 30S subunit. In keeping with such a scenario, hygromycin B inhibits spontaneous reverse translocation, in contrast to the strong stimulation by paromomycin, neomycin, and gentamicin [39].

21.3.2
Target: 30S Body

Streptomycin binds to the body of the 30S subunit and connects rRNA regions from all three domains of 16S rRNA, making contacts with helices h1, h18, h27, h28, and h44; additional contacts are to protein S12 [34]. Streptomycin induces an error-prone conformation of the decoding site (the ''ram'' conformation) in which the binding of tRNAs, including near-cognate ones, is stabilized and GTP hydrolysis by EF-Tu is stimulated [40], resulting in increased misreading. Streptomycin causes a slight inhibition of translocation (twofold), which is less than expected given that the antibiotic stabilizes peptidyl-tRNA binding in the A site nearly 50-fold [19]. The explanation for this discrepancy is that the antibiotic induces a conformation of the 30S subunit that facilitates tRNA–mRNA movement, thereby decreasing the activation energy of translocation and overcompensating the stabilization of peptidyl-tRNA in the A site. In fact, the energetic difference between A-site stabilization and translocation inhibition suggests that streptomycin intrinsically facilitates translocation by about 20-fold. In keeping with this model, it has been shown that the antibiotic accelerates the spontaneous reverse translocation by about the same factor [23]. This similarity suggests that the fundamental features of tRNA movement within the ribosome are comparable for the spontaneous reverse translocation and the forward reaction catalyzed by EF-G, supporting models in which tRNA movement in either direction is driven by Brownian motion.

21.3.3
Target: 30S Subunit, Head Domain

Spectinomycin belongs to the tricyclic aminocyclitol antibiotics. It binds to the minor groove of helix 34 of 16S rRNA near the neck region of the 30S subunit, as shown by crystallographic analysis [34, 41] and chemical protection assays. Translocation is accompanied by structural changes at helix 34 that are induced by EF-G binding to the pre-translocation complex [42], and mutations at the spectinomycin binding site around nucleotide 1191 impair translocation [43]. Spectinomycin inhibits translocation [19, 44] by shifting the conformational equilibrium of the 30S subunit from a rapidly translocating conformation (about $15\,s^{-1}$, $37\,^{\circ}C$) to a slowly translocating one ($0.5\,s^{-1}$), despite a slight overall destabilization of peptidyl-tRNA in the A site. The slowly translocating conformation seems to be intermediate

between PRE and POST [45]. Crystallographic studies have revealed a position of the 30S head that is rotated relative to the 30S body, suggesting a swiveling movement of the head [6]. Head swiveling appears to be important for tRNA movement as it is correlated with spontaneous reverse tRNA translocation [22] and the formation of an intermediate state of the P-site tRNA other than the P/E state [46]. Spectinomycin seems to impair head swiveling by stabilizing one particular state [41], thereby inhibiting translocation.

GE82832 is a secondary metabolite of *Streptosporangium cinnabarinum* that inhibits translation, both *in vivo* and *in vitro*, and has a broad antimicrobial potential [47]. The recent structure determination has revealed that GE82832 is nearly or fully identical to dityromycin [48], an antibiotic isolated from *Streptomyces* that has been described earlier [49]. The biochemical characterization of GE82832 has revealed that the specific target of the compound is the 30S ribosomal subunit and the inhibited step of translation is translocation. The binding site of the antibiotic, as delineated by chemical probing, involves 16S rRNA residues A1324 and A1333, which are protected in the complex, and C1336, which is exposed [47]. These data place the antibiotic in h42 at the top of the 30S head domain, near protein S13. Protein S13, in turn, forms an intersubunit bridge (bridge 1a) with H38 of 23S rRNA and another (bridge 1b) with protein L35, connecting the 30S head domain with the central protuberance of the 50S subunit. An important functional role in controlling translocation has been shown for protein S13, indicating that these bridges are dynamic structures that move during translocation [50]. Thus, binding of GE82832 close to bridge 1a might inhibit translocation by interfering with the concerted movement of the ribosomal subunits required for translocation [47].

21.3.4
Target: Intersubunit Bridge 2a

Viomycin is a cyclic peptide antibiotic that belongs to the family of tuberactinomycins (another family member in clinical use is capreomycin). The antibiotic binds to both 30S and 50S subunits of bacterial ribosomes with a binding site comprising helices h44 and H69 of 16S and 23S rRNA, respectively, which together form the intersubunit bridge 2a. The binding site of viomycin in h44 partially overlaps the binding sites of the aminoglycosides paromomycin and hygromycin B [51]. This position, which is based on crystal structures, is in accordance with the analysis of resistance mutants against capreomycin [52]. Viomycin can bind to ribosomes in the classic nonhybrid/nonrotated state, as shown by the crystal structure of the complex [51], in keeping with a single-molecule FRET study [9]. By contrast, a stabilization of the hybrid/rotated state by viomycin binding has been observed by FRET between fluorescence-labeled ribosomal subunits [53] and in another single-molecule FRET study [54].

Viomycin blocks translocation [19, 55], presumably by stabilizing the flipped-out position of A1492 and A1493 of h44 in the decoding center [51] which form A-minor interactions with the anticodon–codon duplex [35], thereby strengthening peptidyl-tRNA binding in the A site. An inhibition of translocation by an increased affinity

of peptidyl-tRNA in the A site is corroborated by the finding that viomycin, similar to paromomycin, induces reverse translocation even in the presence of EF-G and GTP [23]. Alternatively, it has been postulated that viomycin interferes with the transition from a partially hybrid (P/E, A/A) to the fully hybrid state, that is, with the movement of peptidyl-tRNA from the A/A to the A/P state [45]. However, the latter model does not explain the strong effect of viomycin on reverse translocation.

21.3.5 Target: 50S Subunit, GTPase-Associated Center

Thiostrepton, a thiopeptide antibiotic (related antibiotics are siomycin, nosiheptide, and micrococcin (for review, see [56])), binds to the 50S ribosomal subunit at the L11-binding domain (or "GTPase-associated center"). The antibiotic binds in the cleft between the N-terminal domain of protein L11 and helices H43–H44 of 23S rRNA [57, 58], encompassing nucleotides A1095 and A1067 that are essential for binding. Thiostrepton binding strongly influences the function of EF-G on the ribosome in that both translocation and release of Pi are inhibited, whereas initial binding of EF-G-GTP and the first round of GTP hydrolysis are not impaired [59, 60]. As a consequence, the dissociation of EF-G, which requires Pi release [16, 27], is inhibited, indicating that thiostrepton traps the ribosome-EF-G complex in a slowly dissociating conformation by blocking a rearrangement of the ribosome. The stability of the complex, however, does not seem to be high enough to allow its isolation by nonequilibrium methods, such as ultracentrifugation [61] or gel filtration [62], in accordance with rates of around 1 min^{-1} for turnover GTP hydrolysis by EF-G on vacant ribosomes observed in the presence of thiostrepton [59].

It has been argued that thiostrepton precluded EF-G binding to the ribosome because of a steric clash of the antibiotic with domain 5 of EF-G [62]. The argument is based on the comparison of the crystal structures of a 50S-thiostrepton complex [58] and cryo-EM or crystal structures of ribosome–EF-G complexes stabilized by the GTP analog GDPNP or by fusidic acid, respectively [25, 63]. The problem with these comparisons is that the latter complexes invariably have EF-G in a post-translocation position, with domain 4 reaching into the 30S A site and domain 5, in fact, bound to the L11-binding region in a position where thiostrepton (and other thiopeptide antibiotics) binds. However, according to data from chemical footprinting [59] and/or cryo-EM [26], the positions of EF-G in ribosome complexes stabilized by thiostrepton, both PRE and POST, differ from the complexes stabilized by GDPNP or fusidic acid; in particular, domain 5 of EF-G in the thiostrepton-stabilized complexes is in a position that does not overlap with thiostrepton. The conclusion is that the inhibition of EF-G-dependent translocation and EF-G turnover by thiostrepton is not due to an interference with EF-G binding to the ribosome, which would be incompatible with the observed unchanged activity in GTP hydrolysis, but rather to an inhibition of structural rearrangements of the ribosome–EF-G complex following GTP hydrolysis, as suggested previously [45, 59, 64]. It is not clear whether the inhibition is caused by interfering with the interdomain mobility of L11, as has been suggested on the basis of the structure of the complex [58], or

by occupying the binding site of domain 5 of EF-G, thereby inhibiting the factor from leaving its initial binding position and from promoting ribosome unlocking.

Micrococcin, another thiopeptide, resembles thiostrepton in its chemical structure and also binds to the L11-binding region of the 50S ribosomal subunit [57, 58]. As with thiostrepton, micrococcin strongly inhibits translocation [64]. Unlike thiostrepton, micrococcin has been shown to stimulate, rather than inhibit, turnover GTP hydrolysis by EF-G [57, 61, 64, 65]. On the basis of the structural differences of the micrococcin and thiostrepton complexes, a central role in mediating the different effects of the antibiotics has been attributed to the mobility of the N-terminal domain of protein L11 [58]. Thus, in the micrococcin complex the release of Pi might be stimulated, rather than inhibited, because of a stimulation of L11-dependent recruitment of the C-terminal domain of ribosomal protein L7/12 to the G′ subdomain of EF-G [58]. This contact is important for GTPase activation [66] and Pi release [16]. Pi release, in turn, is a requirement for the rearrangement of EF-G to the conformation – presumably the one of unbound EF-G [67, 68] – that can dissociate from the ribosome [27]. The inhibition, by fusidic acid, of the latter rearrangement is discussed in the following.

21.3.6
Target: EF-G

Fusidic acid, a steroidal antibiotic produced by the fungus *Fusidium coccineum*, is briefly discussed here, although it does not inhibit translocation as such, but rather the turnover of EF-G on the ribosome as well as the function of EF-G in ribosome recycling.

In vitro, fusidic acid can bind to EF-G on the ribosome after GTP hydrolysis, Pi release, and translocation have taken place. The antibiotic binds between domains 1, 2, and 3 of EF-G (Figure 21.3) [25], in accordance with the positions of resistance mutations [69]. The binding affinity is submicromolar [70, 71], as is the minimal inhibitory concentration observed *in vivo* (*Staphylococcus aureus*). Binding of fusidic acid stalls EF-G on the ribosome in the post-translocation position in a conformation that differs from that of the unbound factor, inhibiting the turnover of the factor measured by either GTP hydrolysis or translocation. To achieve an effective inhibition, fusidic acid concentrations of around 100 μM are required, indicating that the fusidic-acid-binding conformation of EF-G on the ribosome, which presumably forms concomitantly with Pi release, is short-lived. This means that EF-G with high probability rearranges to a conformation in which it can dissociate from the ribosome, but cannot bind fusidic acid anymore; at a rate of about $2\,s^{-1}$ this rearrangement is rapid [16]. Therefore, even in the presence of fusidic acids at high concentration, EF-G continues to function through several rounds of elongation (or uncoupled GTP hydrolysis on vacant ribosomes), until fusidic acid binds, and the turnover reaction ceases [27, 60].

At the concentrations of fusidic acid attainable *in vivo*, the inhibition will become effective only after several or many rounds of elongation, and it is, therefore, uncertain whether the inhibition of EF-G turnover in elongation is the primary

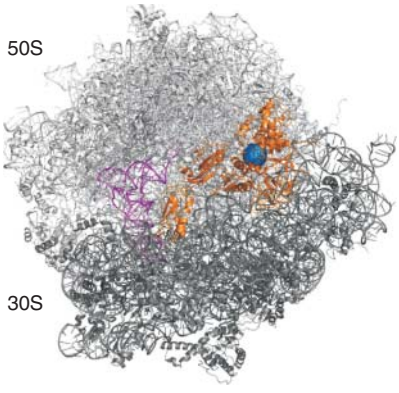

Figure 21.3 Crystal structure of the bacterial ribosome in the POST state with bound EF-G and fusidic acid. The 50S and 30S subunits are depicted in light and dark gray, respectively, EF-G in orange, fusidic acid in blue, and the P-site peptidyl-tRNA in magenta.

target of fusidic acid *in vivo*. An alternative is discussed in subsequent text in the context of EF-G function in ribosome recycling.

21.4 Antibiotics Inhibiting Translocation in Eukaryotes

Although the number of known antibiotics that are active against bacteria is much larger than that of antibiotics active against eukaryotes, there are a few antibiotics that act in eukaryotes (or in both bacteria and eukaryotes) by inhibiting translocation. The few that have provided mechanistic information on translocation are discussed in the following.

21.4.1 Target: 40S Subunit, Decoding Site

Hygromycin B, which is a strong inhibitor of translocation on bacterial ribosomes, inhibits translocation in eukaryotic systems as well [72]. Hygromycin B binds to h44 in the decoding site and interacts with residues (U1406, C1496, U1498) [38] that are conserved in bacteria, archaea, and eukaryotes and whose mutation confers resistance against the antibiotic [73]. Given the high degree of structural conservation of the decoding site, it seems likely that – as in bacterial systems – the inhibitory action of hygromycin B in eukaryotes is due not only to the stabilization of peptidyl-tRNA in the A site [72] but also to an interference with conformational transitions of the small ribosomal subunit that are important for tRNA movement [19].

21.4.2 Target: 60S Subunit, E Site

Cycloheximide, a member of the glutarimide antibiotic family, is well known as an inhibitor of the elongation phase of eukaryotic protein synthesis. The antibiotic

binds to the large (60S) subunit of the eukaryotic ribosome and specifically inhibits the eEF2-dependent translocation [74]. The detailed inhibition mechanism was not known until recently. Surprisingly, during the translation of cricket paralysis virus RNA, which is initiated at an internal ribosome entry site (IRES) without initiator tRNA, the first round of translocation was not inhibited by cycloheximide, whereas subsequent rounds were [75]. This observation suggested that cycloheximide might block the movement of the P-site tRNA (usually initiator tRNA in the first round of elongation) into the E site during translocation. In fact, chemical footprinting experiments revealed that a single cytidine, C3993, in the E site of the 60S subunit of mammalian ribosomes was protected by cycloheximide, or another glutarimide compound of higher potency, lactimidomycin [76]. Apparently, by binding to the 60S E site, the antibiotics prevent the P-site tRNA from entering the P/E hybrid state [1], which is an important prerequisite for translocation to take place [2].

21.4.3
Target: eEF2

Sordarins are a group of antifungal compounds that inhibit protein synthesis by stalling eEF2 on the ribosome, in particular eEF2 from *Candida albicans* and *Saccharomyces cerevisiae*, but not eEF2 from mammalian cells [77, 78]. Thus, sordarin, or its derivatives, carry some potential as antimycotic agents. Sordarin binds to yeast eEF2 between domains 3, 4, and 5 (i.e., in a binding site different from that of fusidic acid on bacterial EF-G) and induces an extensive movement of those three domains relative to domains 1 and 2 [79]. A similar, but slightly different, conformation of eEF2 is induced by sordarin derivatives, indicating that details of the interactions between antibiotics and factor influence the factor's conformation [79]. Sordarin binding to eEF2 is strongly stimulated when eEF2 is bound to the 80S ribosome. On the ribosome, sordarin-stalled eEF2 assumes a conformation in which domains 3–5 are rotated away from domains 1 and 2 [80]. This creates a conformation that differs from that of the unbound eEF2-sordarin complex and may be related to translocation, as it seems to be coupled to conformational changes of the ribosome. These include changes at intersubunit bridges and a movement of the 40S head domain that may be coupled to tRNA movement.

21.5
Antibiotics Inhibiting Ribosome Recycling in Bacteria

Following termination of protein synthesis, that is, hydrolysis of peptidyl-tRNA induced by release factors 1 or 2, the post-termination ribosome carries deacylated tRNA in the P site (P/E state), whereas both E and A sites are unoccupied. In bacteria, the post-termination complex is disassembled by the combined action of EF-G and RRF [81]. Mechanistically, the post-termination complex is disassembled into subunits by EF-G and RRF (Figure 21.4). Subsequent tRNA–mRNA dissociation from the 30S subunit is promoted by IF3, which binds to the 30S subunit and

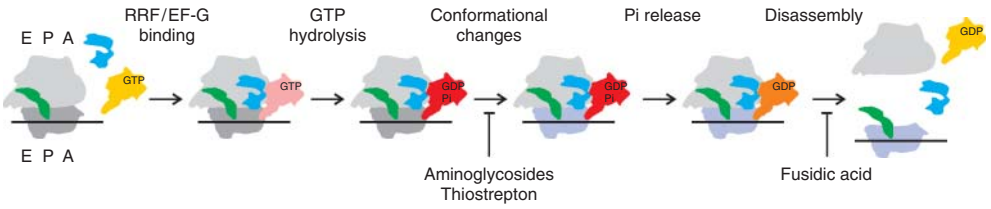

Figure 21.4 Kinetic scheme of ribosome recycling. The post-termination ribosome is depicted with deacylated tRNA (green) in the P/E hybrid state bound to the mRNA in the 30S P site (horizontal line). RRF (blue) and EF-G-GTP bind one after another. Different conformations of EF-G are indicated by different colors. The structural change of the ribosome that is induced by GTP hydrolysis is indicated by different coloring of the 30S subunit. Both factors are released after ribosome disassembly. The dissociation of the tRNA and the mRNA from the 30S subunit following disassembly, as induced by IF3, is not shown.

additionally prevents rebinding of the 50S subunit before the next round of initiation [82–85]. The association of the subunits appears to be weakened by RRF displacing H69, which is engaged in the central intersubunit bridge 2a [37, 86, 87], and the function of EF-G may be to amplify this effect of RRF [88]. The induction by RRF binding of a hybrid, fully rotated state of a ribosome complex with tRNA in the P/E hybrid state has been shown by X-ray crystallography [89].

The two functions of EF-G on the ribosome, that is translocation and ribosome recycling, are distinct, as the disassembly reaction does not imply a translocation-like movement of either RRF or mRNA [83] and strictly requires both GTP hydrolysis and Pi release. By contrast, translocation can take place without GTP hydrolysis, albeit slowly, and does not require Pi release [15, 27]. A number of antibiotics that inhibit the function of EF-G in translocation have been shown to also inhibit recycling or vice versa [27, 90]. These are discussed in the following paragraphs.

21.5.1 Target: Intersubunit Bridge 2a

Aminoglycoside antibiotics, which are strong inhibitors of translocation, also inhibit ribosome recycling with efficacies of around 1 μM (IC50 values of mRNA release) [90]. As discussed earlier, translocation inhibition is attributed to aminoglycoside binding to h44 in the decoding site. However, for several aminoglycosides, a second binding site at H69 seems to be instrumental for the inhibition of recycling. The crystallographic analysis of 30S-RRF and 30S-RRF-aminoglycoside complexes indicates that aminoglycosides (gentamicin and paromomycin), by binding to H69, inhibit the displacement of H69 mediated by RRF [37] and, thereby, the disruption of intersubunit bridge 2a and subunit separation.

Viomycin, as discussed earlier, binds to intersubunit bridge 2a, formed from h44 and H69, and interferes with the classical-to-hybrid rotation of the ribosomal subunits. Its weak inhibition of recycling [90] may be related to interfering with the RRF-mediated change of bridge 2a.

21.5.2
Target: 50S Subunit, GTPase-Associated Center

Thiostrepton binds to the 50S subunit at the L11-binding site. The molecular mechanism underlying the inhibition by thiostrepton of recycling [90] is probably related to the mechanism of translocation inhibition. The antibiotic may block EF-G-induced rearrangements of the ribosome that are required for subunit disassembly either directly or indirectly, via EF-G, by blocking Pi release.

21.5.3
Target: EF-G

Fusidic acid has been reported to inhibit the RRF-EF-G-induced release of mRNA, but not of tRNA, from post-termination complexes [90]. The detailed analysis by a rapid kinetic light-scattering assay with biochemically defined post-termination complexes revealed that fusidic acid inhibits ribosome disassembly into subunits [27]. By binding to EF-G on the ribosome, fusidic acid presumably inhibits a conformational rearrangement of EF-G that takes place after Pi release and is required for ribosome disassembly. Thus, the molecular mechanism of fusidic acid inhibition may be related to the inhibition of EF-G dissociation. Alternatively, the antibiotic may inhibit a structural rearrangement of the ribosome-EF-G complex that is required for disassembly, but not for translocation, for example, an extensive rearrangement of intersubunit bridge 2a, which cannot take place when fusidic acid prevents a conformational change of EF-G that is necessary to drive that rearrangement. The inhibition of ribosome recycling is effective at rather low concentrations of fusidic acid, suggesting that the inhibition of recycling may be the primary effect of the antibiotic and the inhibition of EF-G turnover during elongation, which requires very high concentrations of fusidic acid, the secondary effect.

21.6
Perspective

Many of the antibiotics that have been discussed in this review mainly from a mechanistic point of view are in clinical use. As with all antibiotics, the appearance of strains of bacteria or fungi that have acquired resistances against these antibiotics creates increasingly serious medical problems. The identification of new targets for antimicrobial and antifungal therapy and the development of new inhibitors for these targets are therefore of eminent importance. The ribosome and the partial reactions of protein synthesis, including translocation, are major targets for many natural antibiotics. Thus, these targets will also be useful for the development of new inhibitors. The recent progress in elucidating high-resolution structures of ribosomes and ribosome–antibiotic complexes provides the basis for developing new compounds for therapeutic use against which resistances have not developed yet.

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22
Antibiotics at the Ribosomal Exit Tunnel – Selected Structural Aspects

Ella Zimmerman, Anat Bashan, and Ada Yonath

22.1
Introduction

Ribosomes, which are the target of many antibiotics [1, 2] possess spectacular architecture and inherent mobility, allowing their smooth performance in decoding the genetic information as well as in the formation of the peptide bond and the elongation of the newly synthesized proteins. The site for peptide bond formation (peptidyl transferase center, PTC, is located within a highly conserved pseudosymmetrical region [3, 4] that connects all of the remote ribosomal features involved in its functions, and seems to be a remnant of an ancient RNA machine for chemical bonding [5–10].The elaborate structure of this region and its dynamic properties place the aminoacylated and peptidyl tRNAs in the stereochemistry required for formation of peptide bonds, for substrate-mediated catalysis, and for substrate translocation, namely, for all activities enabling nascent chain elongation.

Adjacent to the PTC is the entrance to an elongated tunnel (Figure 22.1), a universal multifunctional feature of the ribosome, along which the nascent proteins progress until they emerge out of the ribosome. The existence of an internal ribosomal tunnel was proposed first in the 1960s, based on biochemical experiments indicating ribosomal masking of the last to be formed segments of the nascent chains [11, 12]. Nevertheless, at that time and during the following two decades it was widely assumed that growing nascent proteins ''travel'' on the ribosome's surface and are not degraded because they adopt compact helix conformations. In fact, doubts about the mere existence and the universality of the ribosomal tunnel were publicly expressed [13] and studies aimed at supporting this assumption were carried out [14] even after its initial visualization by three-dimensional image reconstructions at rather low resolution in eukaryotic and prokaryotic ribosomes, namely, 60 and 25 Å, respectively [15, 16]. This tunnel was rediscovered by cryo electron microscopy [17, 18] and finally verified when it was clearly observed in the first high-resolution crystal structures of the large ribosomal subunit [19, 20].

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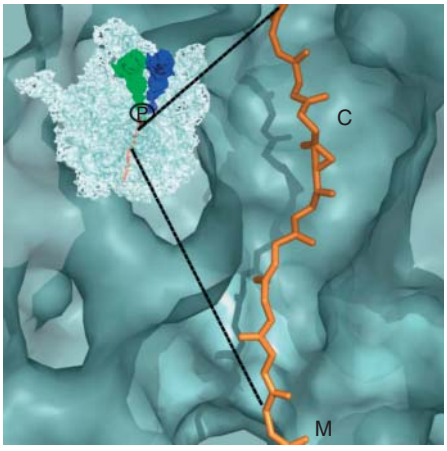

Figure 22.1 The universal nascent protein exit tunnel. Located in the large ribosomal subunit (top left) and extends from the site for peptide bond formation, PTC (P) to the other side of the subunit, the tunnel (highlighted by a modeled polyalanine chain) has a nonuniform shape (seen clearly in the zoomed region). This uneven shape contains a relatively wide crevice (C) alongside a narrow constriction, where members of the antibiotic family macrolides bind (M). A- and P-site tRNAs are shown in blue and green, respectively.

22.2 The Multifunctional Tunnel

Despite its considerable dimensions (about 100 Å in length and up to 25 Å width), uneven shape, and the existence of a wide crevice alongside narrow constrictions (Figure 22.1), tunnel involvement in the fate of the nascent chains was hard to conceive. Therefore, it was originally suggested to be a passive conduit, having a Teflon-like character with no chemical properties capable of facilitating its interactions with the progressively growing nascent chains [21]. However, further studies clearly indicated the significance of the tunnel and its intricate chemical nature and diverse functional roles, such as participation in nascent chain progression and its compaction are currently emerging. Evidence of nascent proteins/tunnel interactions have accumulated (for a review, see e.g., [22, 23]), some of which indicate extensive involvement in translation arrest and cellular signaling. It is conceivable that the interactions of the nascent chains with the tunnel alter the rate of translation elongation and, in extreme cases, lead to translation arrest. Thus, peptide monitoring and discriminating properties can be exploited for optimizing protein targeting and gene expression by small molecules such as tryptophan, arginine, and *S*-adenosyl-methionine [24–29].

The tunnel walls are lined predominantly by ribosomal RNA. The tips of ribosomal proteins L4, L22, and L23 are non-RNA tunnel wall components that are likely to control the tunnel gating and/or trafficking. While protein L23 resides

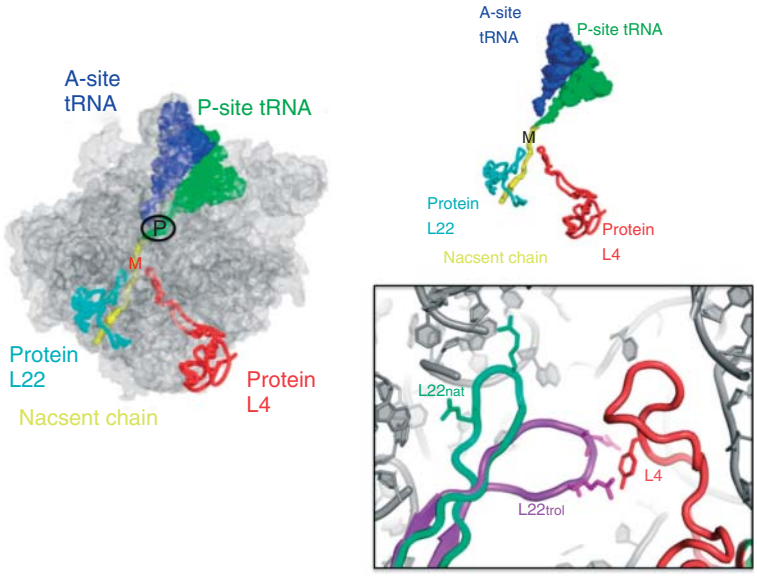

Figure 22.2 Proteins L22 and L4. Left: the positions of ribosomal proteins L4 and L22 in the large ribosomal subunit, shown above the "background," which is the entire large ribosomal subunit (D50S). M is the approximate position of the macrolide binding pocket. Note the proximity of it to the tips of L4 and L22, which, together with L22 elongated shape, allows its indirect involvement in antibiotic resistance as well as its direct participation in elongation arrest and transmission of cellular signals. Top right: same as in the left, but without the large ribosomal subunit. Bottom right: the possible interactions between L4 and L22 in its swung orientation [36]. Only the hairpin tips of the elongated proteins L4 and L22, which reside at the tunnel walls, are shown.

at the tunnel opening and in eubacteria possesses an extended internal loop that appears to have sufficient mobility for controlling the emergence of newly born proteins [30], the hairpin tips of the elongated proteins L4 and L22 reside in proximity to the constrictions of the tunnel wall (Figure 22.2) [20, 31–35] and are involved, mainly indirectly, in antibiotics binding and resistance as well as in nascent chain elongation arrest [36, 37].

Elongation arrest and its mutual impact on cellular processes have gained increased interest in recent years. The discovery of regulatory short nascent peptides that can promote stalling of the macrolide-bound ribosome stimulated studies on sequence-specific interactions of antibiotics with the nascent peptide [38–41] as well as on short peptides that expel macrolide antibiotics from the ribosome while being formed [42–46]; the disparity in the level of macrolides inhibition observed in these cell-free systems has also been investigated [47–50]. Furthermore, it was proposed that in some cases nascent proteins contain arrest segments that may assume specific folds within the tunnel, capable of preventing nascent protein progression along it. It is also conceivable that such semifolded segments could inhibit peptide bond formation or hinder tRNA translocation. Alternatively, arrest

can occur as a consequence of conformational alterations in the tunnel walls that are caused by semifolded segments of the nascent proteins [51, 52].

Strikingly, recent studies indicated that the nascent chains may act as cellular sensors while progressing through the tunnel for regulating membrane protein biogenesis [23, 53, 54]. Indications of possible active tunnel participation in initial nascent chain compaction, leading to semifolded chain segments were accumulated by electron microscopy and single molecule studies (e.g., [54–59]). In addition, indications of distinct conformations, including helical segments of the nascent polypeptide chains, were recently reported within several regions of the ribosomal exit tunnel that have been implicated in nascent chain–ribosome interaction (e.g., [60–63]). Furthermore, crystallographic analysis identified a crevice located at the tunnel wall, where cotranslational initial folding may occur [64]. The currently available observations imply direct interactions between specific residues of the nascent peptide with distinct locations in the ribosomal tunnel in prokaryotes and eukaryotes. These findings indicate that protein L22 appears to have dual functions: it acts as a cellular sensor as well as a progression barrier of the nascent peptide. The C- and N-termini of protein L22, located at the outer surface of the ribosomal particle within the vicinity of the tunnel opening (Figure 22.2), can sense cellular signals and transmit them into the ribosome through the tunnel so that the nascent protein exit tunnel together with intraribosomal regulation processes seem to be responsible for cell–ribosome signaling mechanisms and govern the fate of nascent proteins expression. Furthermore, as revealed in the crystal structure of the large ribosomal subunit in complex with the macrolide antibiotic troleandomycin (see subsequent text and in [36]), the tip of the L22 hair pin, similar to the consequences of troleandomycin binding, is capable of swinging across the tunnel, thereby hampering nascent protein progression, and thus can act as a tunnel gate. In support of this proposition is the finding that the arrest caused by the SecM arrest sequence is bypassed by mutations in the L22 hairpin tip region as well as in the 23S rRNA nucleotides [65] that were mapped to interact with L22 in its swung conformation [36].

22.3
A Binding Pocket within the Multifunctional Tunnel

Simultaneously with the emergence of the first high-resolution structures of the ribosome, the protein exit tunnel was shown to provide the binding pocket of the prominent antibiotics family, the macrolides [34]. Erythromycin, the "mother" of this clinically important antibiotic family [50], was introduced into clinical practice in 1952. It possesses strong bacteriostatic activity against a broad range of gram-positive and several gram-negative pathogens [66]. The location of the erythromycin binding pocket, as in the crystal structures of *Deinococcus radiodurans* 50S/erythromycin complex, can facilitate the obstruction of the tunnel and hamper the progression of the nascent proteins. This agrees with many biochemical experiments [67–69] that showed that erythromycin inhibits, to various extents,

the progression of nascent proteins through the exit tunnel. Indeed, the antibiotic binding to their pocket narrows the tunnel radically, and therefore should hinder the progression of the nascent peptide [34].

A major issue concerning the clinical usefulness of ribosomal antibiotics is their selectivity, namely, their capacity to discriminate between the ribosomes of the eubacterial pathogens and those of eukaryotes. Although prokaryotic and eukaryotic ribosomes differ in size (~2.4 and 4 MDa, respectively), their functional regions, which are the targets for the antibiotics, are highly conserved [70]. Therefore, the imperative distinction between eubacterial pathogens and mammals, the key to antibiotic usefulness, is achieved generally, albeit not exclusively, by subtle structural difference within the antibiotic binding pockets of the prokaryotic and eukaryotic ribosomes. A striking example of discrimination between pathogens and humans is the huge influence played by the minute difference at position 2058 of the rRNA, where the bacterial adenine is replaced by a guanine in eukaryotes. Indeed, this small difference was found to govern the binding of macrolides.

Investigations on the binding modes of the macrolides allowed the identification of the chemical parameters determining the mechanism of action of the various members of this family of antibiotics [31, 32, 34–36, 71–73]. Structural studies deciphered the parameters influencing and fine tuning antibiotic binding [73], revealed the inherent flexibility of tunnel wall components that facilitates remotely acquired antibiotics resistance (see preceding text and in [36]), and shed light on the passage of a distinct subset of polypeptides.

22.4
Remotely Acquired Resistance

Four decades ago resistance to erythromycin was detected in mutants of laboratory strains of *E. coli*, in which proteins L22 and L4 underwent minor modifications. These proteins are located in the vicinity of the macrolide binding pocket [34] and are involved in erythromycin resistance [74], in spite of not belonging to the pocket. Minute sequence alterations in the tip of the hairpin of protein L22 and/or in protein L4, in proximity to the swung L22 (Figure 22.2), were shown to confer erythromycin resistance, without preventing erythromycin binding [75, 76]. Analysis of the structures of an L22-resistant mutant showed that this mutation triggered significant displacements of the RNA components of the tunnel walls (Wekselman *et al.*, work in progress). These rearrangements seem to cause tunnel broadening, so that it can host erythromycin while allowing the progression of nascent polypeptide chain. Interestingly, the influence of L22 conformation on the shape of the tunnel wall was detected also by electron microscopy [77, 78]. Finally, it is interesting to note that remotely acquired resistance seems to be the mechanism for acquiring resistance to antibiotics targeting the PTC, including the pleuromutilins [79].

22.5
Resistance Warfare

Despite the initial, overwhelmingly positive clinical results obtained with erythromycin, this antibiotic was found to be rather sensitive to acidity and hence less suitable for treating stomach infections. These, and similar shortcomings, stimulated the design of semisynthetic antibiotics, such as clarithromycin, roxithromycin, and clindamycin. These also led to the design of new compounds meant to combat with antibiotic resistance that developed about a decade after the beginning of the clinical use of the antibiotic. Indeed, in several cases, enhanced chemical stability, higher inhibition activity (namely, lowering drug concentration), a wider coverage against various pathogens and binding to erythromycin-resistant strains were achieved by the modified macrolides, such as the second-generation azalides, such as, azithromycin [80] and the third-generation ketolides, such as telithromycin [81–83].

An interesting example is azithromycin, one of the world's best selling antibiotics that was designed in the 1980s by researchers at PLIVA, Croatia, with the aim of combating resistance. Its main ring is a 15-membered derivative of erythromycin, obtained by inserting a methyl-substituted nitrogen atom into the 14-membered macrolactone ring (Figure 22.3). Azithromycin is potent against several resistant

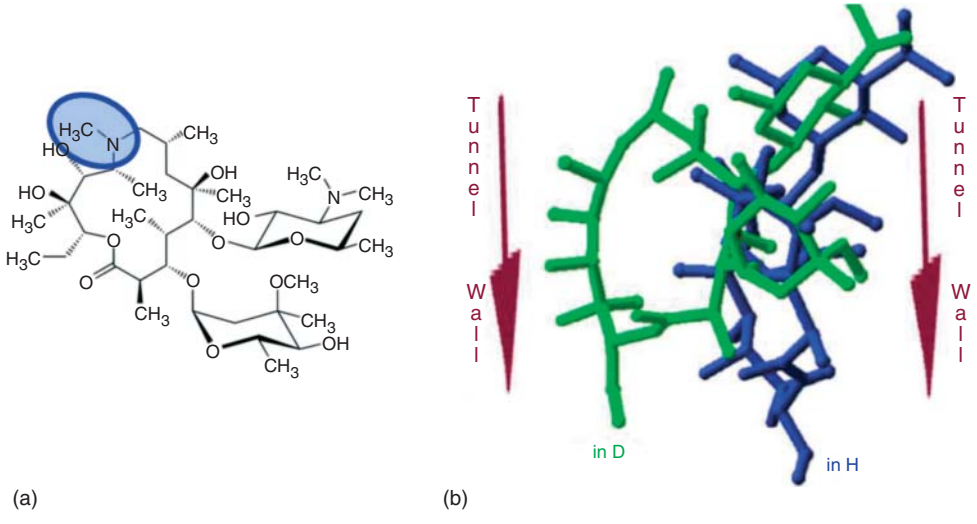

Figure 22.3 Azithromycin. (a) The chemical structure of the macrolide second-generation azithromycin that binds and inhibits erythromycin-resistant strains. This is a 15-membered macrolactone ring, derived from erythromycin by the insertion of a methyl-substituted nitrogen atom (in light blue) into the 14-membered macrolactone ring of erythromycin. (b) The modes of azithromycin binding: across the tunnel to D50S (in green) [72] and along the tunnel in H50S (in blue) in which 2058 is guanine, as in eukaryotes, showing the difference between azithromycin binding to pathogens (D50S) and patient (H50S) models and indicating the consequence in therapeutic effectiveness.

strains, including those with the potentially hazardous A to G mutation at position 2058, as this substitution may result in binding to eukaryotic ribosomes that carry G at this position. However, comparison between the azithromycin binding mode to ribosomes that can serve as pathogen models, namely, of *D. radiodurans* [72] with the binding to a eukaryotic model, namely, the large ribosomal subunit from the *Haloarcula marismortui*, H50S [33] showed clearly that mere binding of an antibiotic compound to the ribosome is not sufficient for obtaining efficient therapeutical effectiveness and indicated that other structural elements of the binding pocket are important for inhibitory activity. Similar observations were made by mutagenesis in the yeast *Saccharomyces cerevisiae* at a position equivalent to *Escherichia coli* A2058, which allows erythromycin binding but does not confer erythromycin susceptibility [76].

22.6 Synergism

Additional attempts aimed at controlling resistance include the development of synergetic antibiotics. An example is the very potent antibiotic called *Synercid*®, a combination of the two streptogramins, dalfopristin and quinupristin, each of which is a rather weak drug, but together they block the PTC as well as most of the tunnel while preventing each other from leaving their binding pockets (Figure 22.4). The impressive synergetic effect of this family can be understood

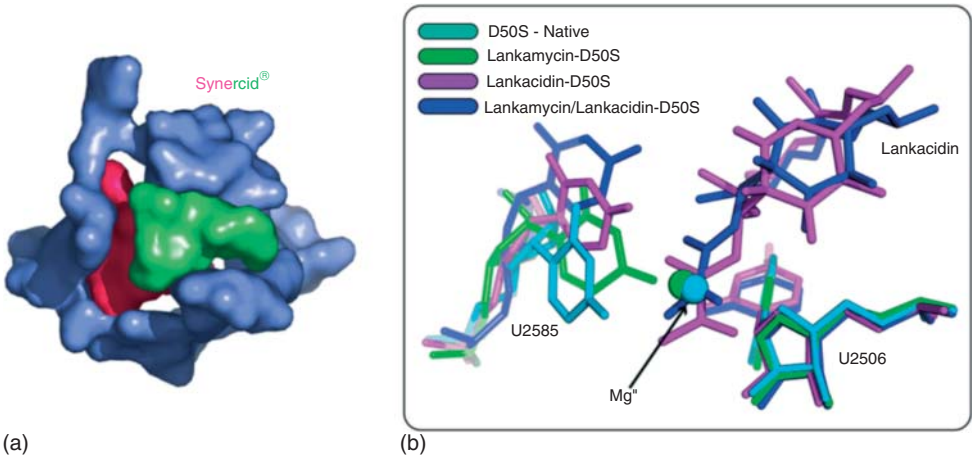

Figure 22.4 Synergism. (a) The two Synercid® components bound to the PTC and tunnel entrance and block them as well as preventing each other from leaving their binding pockets [86]. The tunnel wall is shown in blue. (b) The binding mode of the two components of the lankacidin–lankamycin to D50S [85] and the alterations in the orientation of the very flexible nucleotide U2585 that occur upon binding of the components of this pair.

by examining the mechanism of action of this antibiotic. The two components of the synergetic pair of Synercid bind to the PTC and to the tunnel entrance and displace A2062 and U2585 (Figure 22.4) [84, 85]. Thus, the inhibition is based not only on blocking the tunnel and the entrance to it but also on a dramatic alteration in the orientations of two highly flexible nucleotides, A2062 located at the entrance of the tunnel and U2585, a principal participant in peptide bond formation [3, 84–86].

A similar pair, produced by *Streptomyces rochei*, composed of lankacidin and lankamycin, is expected to be a potential synergistic drug although currently, this pair shows only a modest inhibitory effect on cell growth as well as on cell-free translation. Remarkably, lankamycin binds readily to preformed lankacidin-bound large ribosomal subunits, whereas erythromycin, which has a very similar structure (Figure 22.5 and on the book's cover), disrupts lankacidin binding. The molecular basis for this unexpected difference has been identified [85] and it is likely that it can be exploited for increasing the inhibitory effect of this pair.

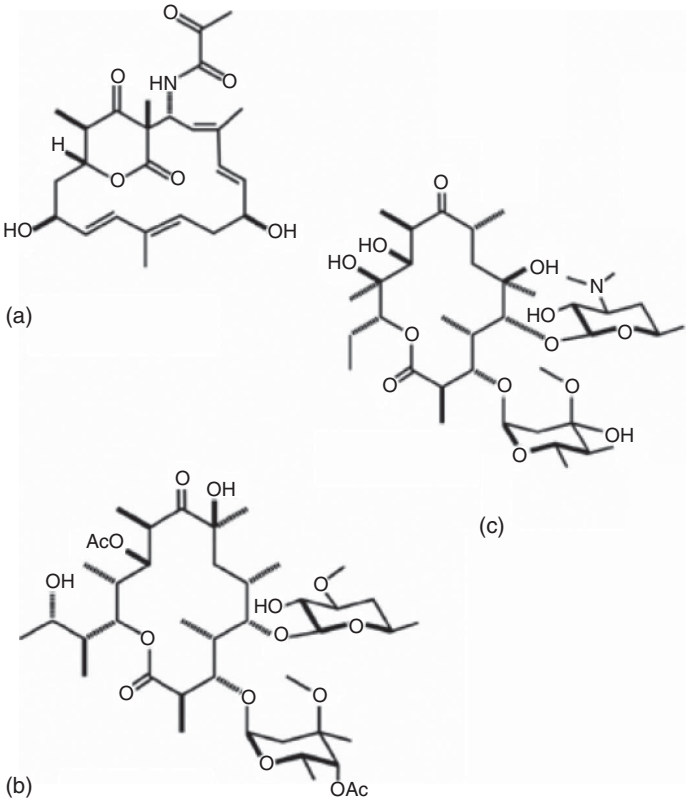

Figure 22.5 The chemical formula of (a) lankacidin C, (b) lankamycin, and (c) erythromycin.

22.7
Pathogen and "Patients" Models

High-resolution structures have provided many clues pertinent to antibiotic drug development. As most eubacteria utilize similar structural principles for antibiotic selectivity and resistance, it is expected that the factors allowing for selectivity should provide powerful tools to understand many of the mechanisms exploited for acquiring resistance. Indeed, the lessons learned from ribosome crystallography concerning combating resistance to antibiotics targeting the ribosome have led to new ideas for antibiotic improvement. However, it should not be forgotten that all of these insights are based on structures of ribosomes from eubacteria that were found to mimic pathogens under clinical-like conditions (e.g., *D. radiodurans, E. coli*, and *Thermus thermophilus*), as so far no ribosomes from genuine pathogens have been crystallized. Consequently, the current observations provided useful clues about common traits, such as modes of actions, details of binding interactions, rationalizations of resistance mechanisms, and the bases for synergism.

Although the currently available structural information is valuable, it seems to be still insufficient for the acute medical challenges. This is because (i) significant variability was detected between binding modes of drugs of the same family (e.g., [36, 71, 87, 88]); (ii) binding pockets contain species specific unique chemical properties that seem to confer resistance; and (iii) in several cases remote interactions are responsible for certain induced fit binding abilities. These enable species discrimination [79], which does not exist within the highly conserved antibiotics binding pockets, and may vary between pathogenic and nonpathogenic bacteria. Combined with the identification of deleterious mutations in rRNA, there is considerable justification to explore ribosomes from the actual pathogenic strains.

The large ribosomal subunit from *H. marismortui* (H50S) can be considered among the currently known high-resolution eubacterial structures that represent suitable models of pathogenic bacteria. Furthermore, in light of the properties that this archaea shares with eukaryotes, in some instances its ribosomes may be considered as a suitable model for patients.

Another example of different binding modes, similar to that of azithromycin binding (Figure 22.3), is observed in crystals of ribosomal complexes with the ketolide telithromycin (Figure 22.6). Thus, even when the nucleotide at the discrimination position for macrolides and ketolides was modified from G2058 (in native H50S) to A2058, as in eubacteria, significant differences were observed in the modes of telithromycin binding to these compared to D50S and to *T. thermophilus* and *E. coli* ribosomes. Importantly, all of the differences in the modes of binding could be rationalized structurally by stacking interactions with tunnel wall components situated in slightly different positions in the various structures (Figure 22.6); this highlights the significant species specificity existing in antibiotic susceptibility and sheds light on the clinical diversity between different pathogens. Importantly, although all macrolides bind to the same binding pocket in a similar manner, some differences in the exact binding modes, which can be explained

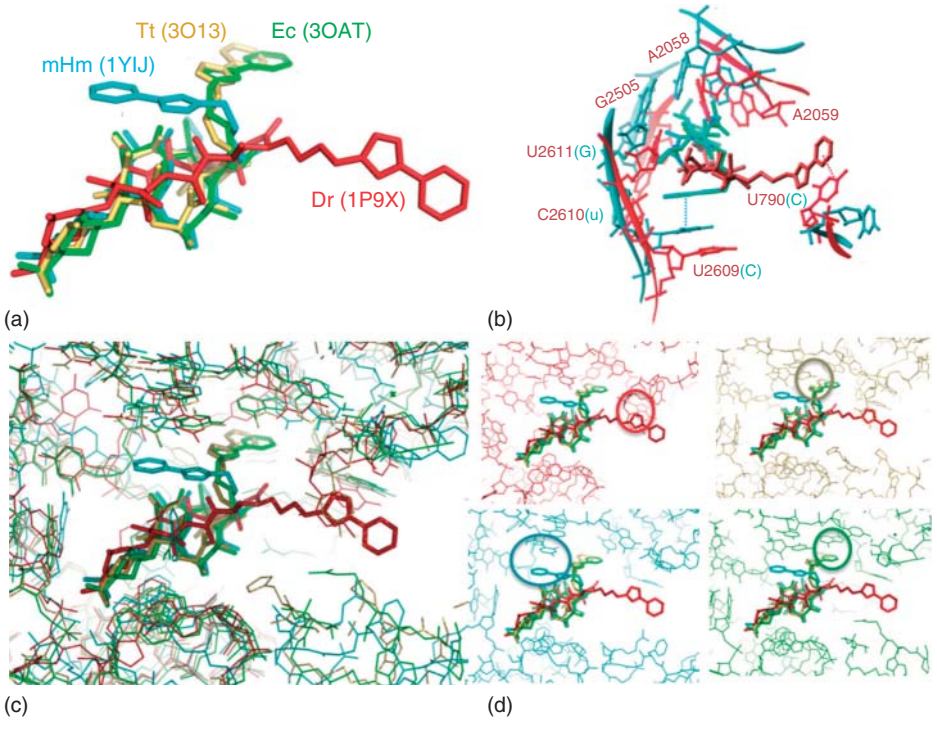

Figure 22.6 Species specificity revealed by the binding modes of the ketolide telithromycin, as observed in crystals of its complexes with various ribosomal particles. In all: color code for the orientations of telithromycin as well as the binding pockets: red: D50S (also called Dr), beige: T70S (also called Tt), green: E70S (also called Ec), cyan: mH50S (also called mHm, namely, a mutant of H50S in which G2058 was replaced by A2058 in order to enable telithromycin binding). (a) All four telithromycin orientations superposed on each other. Note that the macrolactone ring occupies the same position, whereas the long aliphatic arm is extremely flexible, and stretches to different directions, dictated by stacking interactions with the pocket's components. (b) Showing the orientations in the two extreme situations: in D50S and in mH50S, together with the various components of the binding pockets and indicating the stacking by broken lines. (c) All structures within their pockets. (d) The four panels show each of the binding modes within its pocket, with the circle indicating the stacking interactions.

chemically, were identified not only between the various members of this family but also between two erythromycin/D50S complexes [34, 88].

It should not be forgotten that the crystallographic information has shed light on mechanisms for antibiotic function and resistance, although the crystal structures were obtained under conditions barely mimicking the relevant pathogen–host relationships. Thus, *T. thermophilus* grows normally at temperatures that cause disintegration of the antibiotics (namely, $>75\,^{\circ}C$); the entire ribosome from *E. coli* was crystallized without mRNA and tRNA substrates, thus representing an artificial functional state: the archaeon *H. marismortui* grows at elevated temperatures

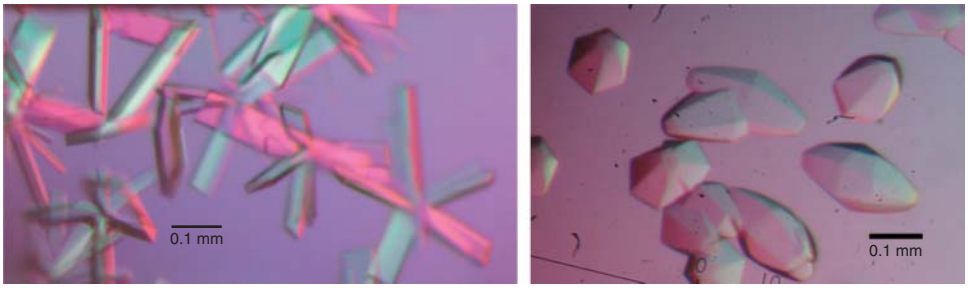

Figure 22.7 Crystals of ribosomes form pathogens. (a) Crystals of the large ribosomal subunits from *Staphylococcus aureus*. (b) Crystals of the small ribosomal subunits from *Mycobacterium smegmatis*, the diagnostic pathogen model for *Mycobacterium tuberculosis*.

in ~3 M KCl, conditions that obviously cannot exist within human or animal cells, and contains features representing eukaryotes and eubacteria, and *D. radiodurans* grows significantly slower than typical bacteria.

In light of this, it is clear that structural information obtained from ribosomes of genuine pathogens should reveal crucial parameters that can be useful for combating resistance. Attempts in this direction are currently under way (Figure 22.7).

22.8 Conclusion and Future Considerations

The rapid increase in antibiotic resistance among pathogenic bacterial strains poses a significant health threat. Hence, improvement of existing antibiotics and the design of advanced drugs are urgently needed. Attempts to overcome antibiotic resistance and increase their selectivity are currently going on, exploiting several strategies including the insertion of moieties that should compensate for the lost interactions of the resistant strains, designing and/or improving natural synergetic pairs, creation of novel compounds possessing inhibitory properties of various levels of potency, and reviving ''forgotten'' antibiotics families.

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23
Targeting HSP70 to Fight Cancer and Bad Bugs: One and the Same Battle?

Jean-Hervé Alix

23.1
A Novel Target: The Bacterial Chaperone HSP70

Drug-resistant microbial pathogens, especially those of nosocomial origin, eventually arise for each antibiotic, by a variety of mechanisms [1]. Development of antibacterial drugs is therefore urgently needed, particularly to battle emerging multidrug-resistant bacteria [2, 3]. Biofilms, an aggregate of microorganisms in which cells adhere to each other on a surface, have also an enormous impact in medicine, as biofilm-grown cells can become 10 to 1000 times more resistant to the effects of antimicrobial agents [4].

A variety of strategies can be conceived: (i) design improved versions of old drugs, (ii) use of bacteriophage therapy, (iii) antivirulence approaches, (iv) therapeutic antibodies [5], (v) apply new screening strategies [6], (vi) find potentiators of traditional antibiotics, and (vii) exploit combination therapy [7]. The most successful example of the latter approach is the combination of the β-lactam antibiotics with β-lactamase inhibitors [8].

Another obvious strategy is to identify novel antibiotics with new modes of action, that is, unveiling new targets and searching for target-selective inhibitors [9]. Recent examples of such targets valid for antibacterial drug discovery are listed in the Table 1 of Ref. [10] and in the Tables 2 and 7 of Ref. [11].

Many effective drugs act via modulation of multiple proteins rather than single targets [12], setting the crucial paradigm of network pharmacology in drug discovery [13, 14]. In other words, the best approach to limit drug resistance would be that of using a single drug affecting multiple, essential targets that are either absent or evolutionarily distant in eukaryotes.

From the work of my laboratory and others, I propose here a novel target that satisfies some of these demands but has received little attention until now, namely, the bacterial chaperone machine HSP70.

The bacterial chaperone HSP70, termed *DnaK* for historical reasons [15] is a molecular machine (Figure 23.1) that functions with two partner proteins, referred to as *co-chaperones*, DnaJ (defined by the presence of a 70-amino acid J

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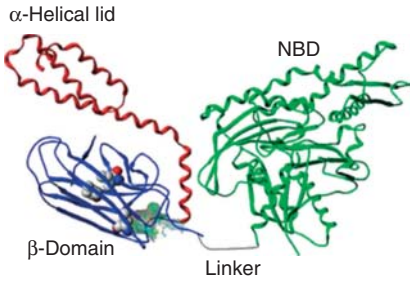

Figure 23.1 DnaK (70 kDa) consists of two domains, that is, a highly conserved amino-terminal ATPase domain of 45 kDa (nucleotide-binding domain (NBD), depicted in green), and a carboxyl-terminal substrate-binding domain of 25 kDa (SBD). The SBD is further divided into a β-sandwich subdomain (depicted in blue) and a α-helical subdomain or lid (depicted in red) that closes over bound substrate. NBD and SBD are connected by a flexible linker region that contributes to allosteric interactions between the two domains. The binding of ATP to DnaK results in an "open" conformation with low substrate affinity but high substrate exchange rate. Upon hydrolysis, the ADP-bound form assumes a "closed" conformation that binds substrate with high affinity and low exchange rate. Allosteric communication between the two domains links nucleotide turnover to the substrate binding and release.

domain motif), and GrpE, a nucleotide adenosine-5′-triphosphate (ATP)/adenosine-5′-diphosphate (ADP) exchange factor [16–20]. They are heat-shock proteins (as all the other bacterial chaperones, except the trigger factor) and fulfill a myriad of functions, summarized as follows:

1) A *de novo* protein folding, that is, a cotranslational folding of ribosome-bound nascent polypeptide chains
2) Protein repair and reversion of aggregation of misfolded proteins, probably mediated via substrate unfolding
3) Protein translocation across membranes
4) Posttranslational quality control to detect and eliminate the proteins irreversibly unfolded, in cooperation with proteases
5) Macromolecular assembly and disassembly (multimeric proteins and nucleoprotein complexes such as the ribosomes)
6) Retro-control of the heat–shock response, which is triggered under stress conditions

This plethora of DnaK-dependent cellular roles fulfilling functions of housekeeping and defense against stress [21] qualify this chaperone as an ideal target to hit *en bloc* several protein factors and metabolic pathways that are clients of DnaK. For example, a major involvement of DnaK has been described in bacterial ribosome assembly [22–24]. Inhibitors of DnaK should therefore hinder ribosome biogenesis in addition to all other DnaK-mediated cellular functions.

Paradoxically, DnaK is not *sensu stricto* essential. Thus, in *Escherichia coli* a Δ*dnaK* null mutant grows at 30 °C under standard conditions in a laboratory setting. But the phenotypes "essential" versus "nonessential" may depend on the specific context in which they are observed [25]. For example, this *E. coli* mutant defective in DnaK

is highly thermosensitive as it does not grow at 39 °C, and it is also impaired in cell division, septation, motility, chromosome segregation, osmotic adaptation, protein secretion, λ and P1 phage replication, and hypersensitive to some antibiotics [26–29]. In this perspective, a synergy between levofloxacin and a DnaK inhibitor (CHP-105) has been reported [30]. Disruption or forced downregulation of DnaK in the bacterial pathogens *Staphylococcus aureus* [31, 32], *Streptococcus mutans* [33], and *Listeria monocytogenes* [34, 35] leads to thermosensitivity, increased susceptibility to oxidative (disinfectant) and antibiotic stress conditions, and impaired biofilm formation. The DnaK/DnaJ chaperone machinery is essential for the invasion of epithelial cells by *Salmonella enterica* [36] and *Francisella tularensis* [37], for efficient phagocytosis of *L. monocytogenes* with macrophages [34], and for the replication of *Brucella suis* in macrophages [38]. Disruption of the *dnaK* gene of the opportunistic pathogen *Streptococcus intermedius* causes attenuation of cytotoxicity [39]. Inactivation of DnaJ in *Campylobacter jejuni* results in a mutant incapable of colonizing chickens [40]. The sum of these results points unambiguously to the essential role of bacterial molecular chaperones in bacterial infection and virulence. Indeed, a compound named BI-88E3 interacting with or near the substrate-binding domain of DnaK is capable of inhibiting growth of *E. coli* and *Yersinia pseudotuberculosis* [41].

On the other hand, members of the HSP70 family are ubiquitous, universal, and highly conserved throughout evolution. The *E. coli* DnaK is approximately 50% identical to human HSP70s [42, 43]. Can it be selectively targeted for antibacterial applications, despite this high sequence homology? A positive answer is provided by the example of pyrrhocoricin, a proline-rich antibacterial peptide that binds to DnaK but not to the human HSP70 [44–46]. More generally, proline-rich antimicrobial peptides are nontoxic to mammalian cells (mice, human cell lines), as they do not pass through the membrane [47, 48]. Also, a domain swapping experiment between the ATPase domains and the substrate-binding domains of rat HSP70 and *E. coli* DnaK has revealed that the substrate-binding domain of DnaK is essential to fulfill the specific functions of this protein (growth of *E. coli* at high temperature and λ bacteriophage replication) [49]. Even in bacteria, the same domain swapping experiments between *Bacillus subtilis* and *E. coli* have shown that the ATPase domain and the substrate-binding domain carry species-specific functional units [50]. The DnaK chaperones from the archeon *Methanosarcina mazei* and *E. coli* have also different substrate specificities [51].

Finally, it is evident that DnaK and eukaryotic HSP70s differ in their abilities to interact with a defined set of co-chaperones, and have their own network of cooperating and competing factors [52].

Nevertheless, considering the variety of natural and synthetic compounds that have been characterized as inhibitors of the eukaryotic HSP70s (Section 23.3), it would be of great interest to assay their potential antibacterial capacities. Indeed, some of them have already been known for a long time as antibiotics, such as 15-deoxyspergualine [53], gentamicin [54], novobiocin [55], and geldanamycin, which is considered as the prototype of the chaperone HSP90 inhibitor [56] (Section 23.5).

A reason for which DnaK has been very scarcely exploited until now as a target is possibly the lack of a simple assay to monitor the *in vivo* activity of this chaperone. Thus, in Section 23.2 an example of such an assay is described.

23.2
An *In vivo* Screening for Compounds Targeting DnaK

E. coli β-galactosidase, the product of the *lacZ* gene, is a homo- tetramer (1023 amino acid residues in each monomer). A spontaneous small internal deletion termed *lacZ*Δ*M15* that does not disturb the open reading frame has been isolated, and its product (the M15 protein (116 kDa) that lacks residues 11 through 41) is able to form only an inactive dimer [57]. The missing peptide (7.5 kDa) is termed *α-fragment* or *α-donor* because it has the interesting property of allowing the dimerization of the M15 protein dimer (now termed *α-acceptor* or sometimes *ω-fragment*) to form the enzymatically active tetramer [58–60] according to a multistep process determined *in vitro* [61]. This is the most well-known example of protein fragment complementation in which two segments of a protein associate noncovalently to form a functional structure. The α-complementation of β-galactosidase occurs both *in vivo* and *in vitro*. As DnaK catalyzes specific inter- and intramolecular protein interactions, it was reasonable to think that DnaK should participate in the α-complementation of β-galactosidase, a paradigm for the formation of a quaternary structure. This was indeed the case [62]. We have shown by comparison between a wild-type *dnaK* strain and either a *dnaK756-ts* (Figure 23.2a) or a Δ*dnaK* null mutant (Figure 23.2b) of *E. coli* grown at a semipermissive temperature that functional DnaK is necessary to produce an enzymatically active α-complemented β-galactosidase. Plasmid-driven expression of wild-type DnaK restores the α-complementation in these mutants, but also stimulates it in a wild-type *dnaK* strain. This role of DnaK in α-complementation, either direct or indirect, provides an easy and original phenotype: blue versus colorless bacterial colonies on plates containing the chromogenic substrate X-Gal, as well as the measurement of β-galactosidase activity in bacterial crude extracts, to detect functional changes in DnaK, thus providing a reliable tool to monitor the activity of DnaK *in vivo*, and therefore to screen routinely for potential inhibitors or compounds modulating its activity. The detailed procedure, the possible pitfalls of this methodology, and the ways to circumvent them have been described [22].

23.3
Drugging HSP70

Cancer cells are subjected to many external stress conditions (hypoxia, nutrient deprivation, and exposure to chemotherapeutic agents) and to internal stresses following the accumulation of incorrectly folded proteins. The stress response pathway they develop is mediated by molecular chaperones such as HSP70 and

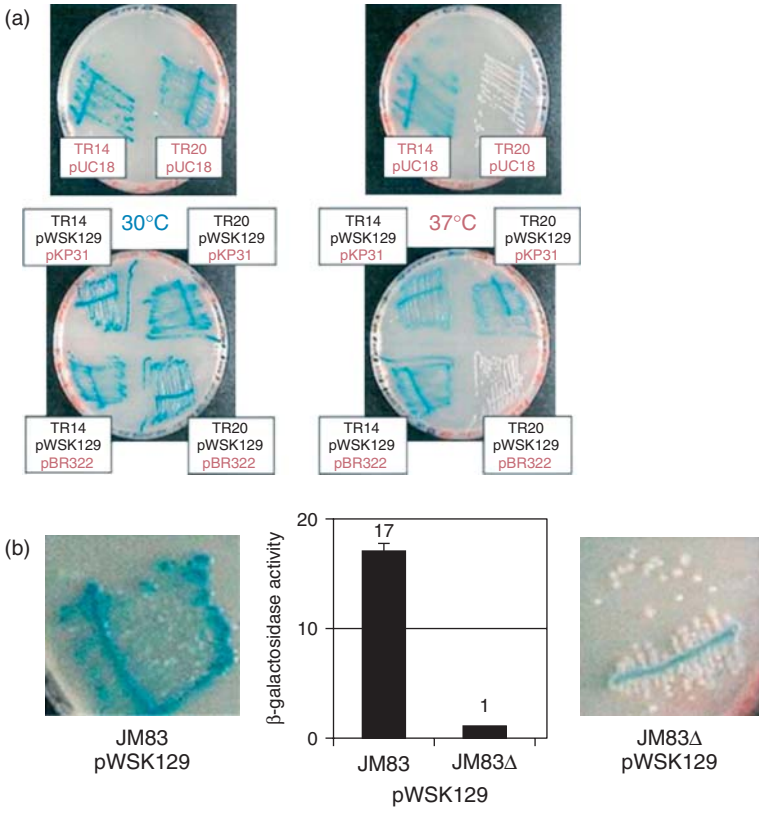

Figure 23.2 (a) α-Complementation of β-galactosidase in wild-type (*dnaK*$^+$) and *dnaK-ts* mutant strains plated at 30 °C (top and bottom left) and 37 °C (top and bottom right). The *E.coli* strains TR14 (*dnaK*$^+$) and TR20 (*dnaK756-ts*), both harboring a chromosomal copy of the *lacZ*Δ*M15* allele, were transformed with plasmid pUC18 (top) or plasmid pWSK129 (bottom) and streaked on plates containing 100 μg of X-Gal per ml. pUC18 (a high copy number plasmid) and pWSK129 (a low copy number plasmid) express both the LacZ α-peptide. Plasmid pKP31, which expresses DnaK, and the control empty vector pBR322 are compatible with pWSK129. (b) α-Complementation of β-galactosidase at 30 °C in a strain that contains a disrupted *dnaK* gene. Strains JM83 (*dnaK*$^+$) and JM83Δ (Δ*dnaK52*: cm^R), both harboring a chromosomal copy of the *lacZ*Δ*M15* allele and carrying the plasmid pWSK129 that expresses the LacZ α-peptide, were streaked on a plate containing X-Gal. The same bacteria were also grown in liquid cultures at 30 °C and harvested at the beginning of the stationary phase. β-galactosidase activity was measured in the bacterial crude extracts.

HSP90. Increased expression of HSP70s is commonly associated with the malignant phenotype. HSP70s promote malignant cell survival by inhibiting apoptosis at multiple points [63]. Therefore, they have long been considered as critical targets for cancer therapy and their downregulation or selective inhibition as a valuable anticancer strategy [64].

The first small molecule inhibitor of HSP70 described is the natural product 15-deoxyspergualin originally identified as a potential antibacterial agent [53, 65–67].

Other chemical agents that inhibit HSP70 function by binding to its substrate-binding domain are geranylgeranylacetones [68], fatty acylbenzamides [69], 2-phenylethynesulfonamide or pifithrin-μ [70], BI-88E3 [41], gentamicin [54], pyrrhocoricin [45, 46], its dimeric analog CHP-105 [30], some other pyrrhocoricin derivatives [71], and some peptides [47, 72–74] and aptamers [75].

Some other chemical agents inhibit HSP70 function by binding to its nucleotide-binding domain such as some adenosine derivatives [76, 77], 3′-sulfogalactolipids [78], some pyrimidinone derivatives [79–81], MKT-077 [82], and methylene blue [83]. D-peptides inhibit DnaK by binding to DnaJ [84] and the flavonoid myricetin blocks the DnaJ-stimulated allosteric regulation of DnaK [85–87].

Recent reviews describe progress made in developing such pharmacological modulators of HSP70 [88–90].

23.4
Cooperation between the Bacterial Molecular Chaperones DnaK and HtpG

Another important molecular chaperone that recognizes a metastable structure in its substrates rather than hydrophobic stretches or amino acid sequences is HSP90 (its prokaryotic homolog is named HtpG, Figure 23.3). In eukaryotes, HSP90 is responsible for the maturation of approximately 200 client proteins, several of which are *bona fide* oncoproteins and key regulators of cell growth.

To accomplish its function, HSP90 collaborates with HSP70 and a multitude of accessory co-chaperones to form large dynamic complexes, sometimes called *foldosome*.

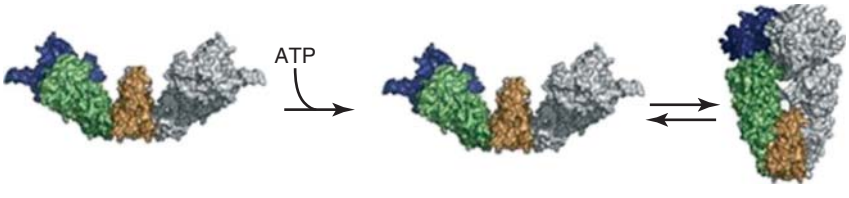

Figure 23.3 HtpG is a dimer. Each monomer has an N-terminal domain shown in blue, a middle domain shown in green, and a C-terminal domain, shown in brown, which is essential for dimerization. The second identical monomer is shown in gray. In the nucleotide-free (*apo*) state, HtpG adopts an open conformation that has a large cleft accessible for client protein binding. Once ATP is bound, an equilibrium is established between the open state and a compact closed or ''tense'' state in which the N-terminal domains are transiently associated. A client protein bound in the absence of ATP undergoes some rearrangement during the passage through the ''tense'' and functional state of HtpG in the presence of ATP. The N-terminal dimerization is required for ATP hydrolysis. Then, dissociation of ADP restores the open conformation. *Source*: Reprinted with permission from Krukenberg *et al*. [91].

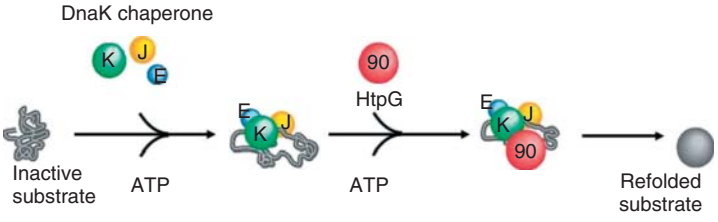

Figure 23.4 Model for protein reactivation by the collaborative activities of the DnaK chaperone machine and HtpG. **K** = DnaK, **J** = co-chaperone DnaJ, **E** = co-chaperone GrpE, **90** = HtpG. *Source*: Reprinted with permission from Genest *et al.* [96].

HtpG, the HSP90 homolog in *E. coli* [92], has received little attention so far because it is dispensable: the *htpG* deletion mutant is viable [93] and is similar to its wild-type parent in its ability to survive starvation [94] but it grows poorly at high temperature. However, HtpG as well as ClpB, another molecular chaperone, participates in protein folding in heat-shocked *E. coli* cells, probably through expanding the ability of the DnaK-DnaJ-GrpE team to interact with newly synthesized polypeptides [95]. Indeed, HtpG and DnaK were shown to interact in a genome-wide screen and in a bacterial two-hybrid assay [96]. They collaborate in remodeling the client protein [96] but unlike DnaK, which binds to the nascent polypeptide chains, the association with HtpG occurs at a later stage of the substrate protein folding process (Figure 23.4). Another example of such a process is the α-complementation of β-galactosidase (Section 23.2), which depends on HSP90 in yeast [97].

In the cyanobacterium *Synechococcus*, HtpG, which is essential for growth at 45 °C [98], stabilizes the assembly of large protein complexes called *phycobilisomes* [99]. The overexpression of HtpG was also found to suppress the thermosensitive mutations of *secY* and *FtsH* in *E. coli* [100], but only one protein substrate of HtpG, the 50S ribosomal protein L2, has been formally identified so far in *E. coli* cultured in minimal medium at 45 °C [101]. Therefore, even though HtpG structure has been analyzed extensively [91, 102, 103], its function is yet elusive.

However, the search for HSP90 inhibitors as potential antimicrobials remains an important goal because of (i) the role of HSP90 in the development of many protozoan parasites [104–107] and fungal pathogens [108, 109] and (ii) the simultaneous attack of multiple cellular pathways by an inhibitor targeting HSP90.

Thus, it was recently shown that cyclic lipopeptide antibiotics such as colistin and polymyxin B target HtpG to abolish its chaperone function [110].

23.5 Drugging HSP90

Geldanamycin is the prototype of the inhibitors of HSP90 [56]. Many other chemical agents targeting HSP90, either natural, such as gambogic acid [111], or synthetic [112–115] have been recently reported.

Others agents may be found in recent reviews [116–120].

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