Antibiotics Targeting Ribosomes: Crystallographic Studies

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Abstract: Resistance to antibiotics is a major problem in modern therapeutics. Ribosomes, the cellular organelle catalyzing the translation of the genetic code into proteins, are targets for several clinically relevant antibiotics. The ribosomes from eubacteria are excellent pathogen models. High resolution structures of the large and small ribosomal subunits were used as references that allowed unambiguous localization of almost a dozen antibiotic drugs, most of which are clinically relevant.

Analyses of these structures showed a great diversity in the antibiotics' modes of action, such as interference with substrate binding, hindrance of the mobility required for the biosynthetic process and the blockage of tunnel which provides the path of exit for nascent proteins. All antibiotics studied by us were found to bind primarily to ribosomal RNA and, except for one allosteric effect, their binding did not cause major conformational changes.

Antibiotics of the small ribosomal subunit may hinder tRNA binding, decoding, translocation, and the initiation of the entire biosynthetic process. The large subunit agents may target the GTPase center, interfere with peptide bond formation, or block the entrance of the nascent protein exit tunnel. The overall structure of the peptidyl transferase center and the modes of action of the antibiotic agents indicate that the ribosome serves as a template for proper positioning of tRNAs, rather than participating actively in the catalytic events associated with the creation of peptide bonds.

Key words: ribosomes, antibiotics, macrolides, tetracycline, edeine, chloramphenicol, clindamycin

1. ANTIBIOTICS AND PROTEIN BIOSYNTHESIS

In rapidly growing bacterial cells, the translation of the genetic code into polypeptide chains consumes up to 80% of the cell's energy and the components involved in this process constitute about half of cellular dry weight. This fundamental life process involves the participation of more than a hundred components. Among them is the ribosome, the largest known macromolecular enzyme. This universal intracellular ribonucleoprotein particle translates the genetic code, in the form of mRNA, into proteins by the sequential polymerization of amino acids.

Ribosomes of all species display great structural and functional similarities. They are composed of two independent subunits of unequal sizes, which associate upon initiation of protein biosynthesis. In prokaryotes, they have molecular weights of 0.85 and 1.45 MegaDa, and are termed 30S and 50S, respectively, in accordance with their sedimentation coefficients. The major component of the ribosome is the ribosomal RNA (rRNA). The 30S subunit consists of one RNA chain (16S) that is composed of about 1500 nucleotides, and some 20 proteins, whereas the 50S subunit consists of two rRNA chains (~3000 nucleotides), termed 23S and 5S, and about 35 proteins.

The smaller subunit has key roles in the initiation of the translation process, in decoding the genetic message, in discriminating against non- and near-cognate amino-acylated tRNA molecules, and in controlling the fidelity of codon-anticodon interactions. The larger subunit contains the peptidyl transferase center, the site where peptide bond synthesis occurs, a process that involves addition of amino acids to the nascent polypeptide chain. Although a detailed mechanism for peptide bond formation has been proposed [1], neither it nor the structural basis of this vital biochemical process have yet been fully elucidated [2-5].

Upon initiation of the process of protein synthesis, the two ribosomal subunits associate to form 70S ribosomes that perform protein synthesis, utilizing amino acids brought to it by amino-acylated tRNA molecules. Within the ribosome there are three binding sites for transfer RNA (tRNA), designated the P (peptidyl), A (aminoacyl) and E (exit) sites, which are partly located on both the small and

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1568-0053/02 $35.00+.00 © 2002 Bentham Science Publishers Ltd.

large subunits. The anticodon loops of the three tRNA molecules bind to the small subunit, whereas the acceptor stems bind to the large subunit. Both subunits work together to move all three tRNAs molecules and the associated mRNA chain by precisely one codon with respect to the ribosome, in a process called translocation. The entire process also depends on an energy source, the hydrolysis #166 of GTP, and several extrinsic cellular protein factors.

Antibiotics are natural or man-made compounds, designed to interfere with bacterial metabolism and eliminate bacteria by inhibiting the biosynthesis of protein, RNA, DNA or cell-wall components [see appendix I]. They are extremely versatile in their modes of action. Their main bacterial targets are cell-wall biosynthesis (ß-lactamases and vancomycin), protein biosynthesis (mainly macrolides, cyclines, aminoglycosides, and oxazolidinones) and DNA replication and repair (fluoroquinolones).

About 40% of the known antibiotics interfere with protein biosynthesis. The ribosome is one of the main binding targets for a broad range of natural and synthetic antibiotics. Structurally diverse natural as well as synthetic compounds efficiently inhibit ribosomal function [6,7]. Theoretically the ribosome offers multiple opportunities for the binding of small compounds, but practically all the known drugs utilize only a few sites. Biochemical information about binding and action of antibiotics on the ribosome has been accumulated for almost four decades.

Sites of antibiotic-ribosome interactions have been deduced by the identification of the mutations or the enzyme-driven modification that occurred as a consequence of binding of the antibiotic agents or, alternatively by chemical footprinting. Structural information on antibiotic binding modes was first obtained by NMR studies, using complexes of antibiotic agents with fragments or models of selected regions of ribosomal RNA [8,9]. These observations illuminated many aspects of the action of the ribosomal antibiotics, but the lack of three dimensional structures of complexes of antibiotics and the ribosome severely hampered the understanding of the modes of action.

Recently, high resolution structures were determined of the two ribosomal subunits, suitable to serve as pathogen-models, each from a different eubacterial source. Using them as references allowed unambiguous localization of several antibiotics [10-13]. Co-crystals were grown, each containing a complex of one of the ribosomal subunits and an antibiotic agent at a clinically relevant concentration. Alternatively, crystals of ribosomal particles were soaked in solutions containing antibiotics at clinically relevant concentrations. In most cases the co-crystals of antibiotics and the ribosomal subunits yielded crystallographic data of quality that was sometimes better than that obtained from crystals of free particles [10,13], presumably because the antibiotics reduce internal motions of flexible regions and increase homogeneity.

Among the known structures of complexes of ribosomal subunits with antibiotics, seven of the antibiotics are of clinical relevance, while two currently have no therapeutic use. Here we do not intend to cover the vast amount of biochemical and medical knowledge of all ribosomal antibiotics. We do, however, review the current state of the structural research on ribosome-antibiotics complexes. We describe the main structural elements of the ribosome, point out logical targets for antibiotics, show examples resulting from our studies and highlight aspects that may be useful for rational drug design.

2. PATHOGEN MODELS FOR CRYSTALLOGRAPHIC STUDIES

All clinically relevant antibiotics are targeted against bacterial pathogens. Hence, structures of complexes of the antibiotic drugs with ribosomes of pathogens or of their models, are essential for the understanding of the antibiotics' modes of action. However, not all ribosomes can be crystallized. So far the key to the progress of ribosomal crystallography has been the availability of crystals suitable for studies at high resolution. Noteworthy is the fact that despite extensive searches for suitable ribosomal sources over more than two decades, only three types have so far led to high resolution structures. These include one of the small subunit from *Thermus thermophilus*, T30S [14,15] and two from the large subunit. One from the archaeon *Haloarcula marismortui*, H50S [16], and the other from the mesophilic eubacterium, *Deinococcus radiodurans*, D50S [17]. The reasons for the dramatically low yield stem from the flexibility of the ribosomes, their multi-conformational nature and, most importantly, the tendency of all ribosomes to deteriorate. Indeed, ribosomes from relatively robust organisms were found to deteriorate less during preparation, thus providing less heterogeneous starting materials for crystallization.

Many ribosomal antibiotics exert their inhibitory effects on eubacteria similar to their modes of action on the known pathogenic bacteria. Ever since the ribosome was characterized as the cellular organelle performing the translation of the genetic code into proteins, the ribosome from *Escherichia coli* has been the preferred source for biochemical, functional and genetic studies. Experience shows that biomedically, pharmacologically and industrially relevant bacterial strains are usually eubacteria, and the ribosomes of *E. coli* were also found suitable as a pathogen model. However, *E. coli* is not one of the three sources that have yet led to high resolution structures of ribosomal particles.

Although *T. thermophilus* and *D. radiodurans* are gram-positive and *E. coli* is gram-negative, the ribosomes of these two eubacteria share extensive similarity with that of *E. coli* [18]. Indeed, *T. thermophilus* and *D. radiodurans* served as suitable pathogen models. Currently structures of complexes of T30S with seven different antibiotics [10-12], and of D50S with five different agents [13] are available. In contrast, the ribosome of the archaeon *H. marismortui* contains features resembling both eubacteria and eukaryotes. Hence it constitutes a sub-optimal model for pathogens. Furthermore, most halophilic ribosomes are resistant to many clinically relevant antibiotic agents, although thorough screening detected a few ribosomal antibiotics with some

inhibitory effects. Among these, only thiostrepton and the universal agents puromycin, sparsomycin, edeine and pactamycin act at a concentration range similar to that found effective for therapeutically use [19].

3. THE RIBOSOME AND ITS GLOBAL ORGANIZATION

The overall structures of both ribosomal subunits are shown in Fig. (**1a**) and described below. The two subunits differ in size, shape and global organization. Whereas the small one is made of distinct structural domains, the core of the large one appears more compact. RNA dominates most of the ribosomal functional activities in both subunits. Proteins are located mainly at the periphery and are believed to stabilize the structure by their long tails that penetrate into the RNA core. These, as well as the globular domains of the ribosomal proteins may assist in the assembly of the particles. Tails that point toward the exterior, however, act like tentacles for enhancing the binding of non-ribosomal factors that participate in protein biosynthesis [12,20,21]. Both subunits contain various functional centers as potential binding sites for antibiotics. The diverse nature of these sites is reflected in the diversity of the modes of action of the ribosomal antibiotics, as seen below.

3.1. The Small Ribosomal Subunit

The high resolution structure of T30S was determined independently by two groups [14,15], and found to be very similar. The structure determined by us is shown in Fig. (**1a**). It contains all morphological features seen in cryo-EM reconstructions, with traditional subdivisions that comprise a "head", a "neck" and a "body" that contains a "shoulder" and a "platform", all radiating from a common junction.

A rather flat surface of the 30S subunit faces the 50S subunit in the complete 70S ribosome. It contains two of the three main helical segments of this subunit, running parallel to the vertical axis of the body: helix H44, in the middle and the main part of the shoulder, on the left. These longitudinal helical elements are likely to transmit structural re-arrangements, correlating events at the particle's far ends with the cycle of mRNA translocation at the decoding region, located at the middle of the meeting point between the body and the neck.

Transverse features, placed like ladder rungs between the longitudinal helical features link them together. Principal among these transverse helices is an inclined lune extending from the shoulder that serves as the lower end of the platform. The head that interacts with mRNA, elongation factor G, tRNA and several antibiotics, joins the body through a slender connection, made of a single RNA helix. It contains most of the 3' region of the 16S RNA. It consists mainly of short helices, arranged in two hemispheres, with a longer helix, serving as the bridge between them.

The highly structured shoulder consists of a co-stack of over 120 Å long helical elements. A non-covalent body-head connection formed by the shoulder and the lower part of the head, is the feature that designates the entrance to the mRNA channel. This connection, called latch [14] facilitates mRNA threading and provides the special geometry that guarantees processivity and ensures fidelity. It controls the entrance to the mRNA channel by creating a pore of varying diameter and its relative location may be dictated by the head twist.

The decoding region is remarkably conserved. In accord with the universality of the decoding process. It contains features from the upper part of the body and the lower part of the head. Among these features are the 3' and 5' ends of the 16S RNA and the switch helix H27. The most prominent feature in the decoding center is the upper portion of H44, which forms most of the intersubunit contacts in the assembled ribosome. Its upper bulge bends towards the neck and forms the three binding sites for tRNA molecules. The switch helix, H27, which packs groove-to-groove with the upper end of H44, can undergo changes in its base-pairing scheme that may induce global conformational rearrangements [22]. The locations of the tRNA molecules on the small subunit were determined by superposition of the structures of the complex of the entire ribosome with three tRNA molecules, determined at 5.5 Å resolution [23]. An important conclusion from this placement, which could be drawn even at the early stages of structure determination, is that the anticodon loops of the A- and P-site tRNAs and the codon segments of the mRNA do not contact any ribosomal proteins. Thus, the configuration of the 16S RNA determines the arrangement of the interacting elements and enforces precision in codon-anticodon interactions.

3.1.1. Conformational Mobility of the Small Ribosomal Subunit

The ribosome is a precisely engineered molecular machine performing an intricate multi-step process that requires smooth and rapid switches between different conformations. The large subunit, which in general has a rather compact architecture, contains structural elements that can undergo local rearrangements. The small subunit that is built of loosely attached domains allows for more global motions required for its function. Analysis of the 30S structure led to suggestion of an interconnected network of features leading to concerted movements during translocation. The major contributors to this conformational mobility of the small subunit are the head and the platform.

Head mobility was confirmed by molecular replacement studies, performed with data collected from the crystals of T30S that diffract to low resolution, together with the high resolution model. The pivotal point for this movement may be at the connection between the head and the neck, rather close to the binding site of the antibiotic spectinomycin that is known to trap a particular conformation of the head and thus hamper its twist [10]. The entrance to the mRNA channel will encircle the message when a latch-like contact closes and contributes to processivity and fidelity. The upper boundary of the mRNA channel is made by the head helix H34, and its relative location is dictated by the head twist. The motion of the head is associated with different latch conformations, guaranteeing processivity and ensuring fidelity and governing the accurate setting of the reading frame.

The global motions associated with translocation include a displacement of the platform. The solvent side of the platform contains the 3' end of the 16S RNA, a highly flexible region that pairs with the trigger sequence in the mRNA to create a critical conformation for the initiation of protein biosynthesis, the step that governs the accurate setting of the reading frame. The solvent side of the subunit is where most of the globular domains of the subunit's proteins are located. Most of them are involved in maintaining the accuracy and maximizing the efficiency of the 30S subunit's tasks. Others, like proteins S18 and S7, assist in the initiation and the translocation processes.

3.2. The Overall Structure of the Large Ribosomal Subunit

The traditional shape of the large ribosomal subunit was determined by electron microscopy [24,25]. The core of the large ribosomal subunit is built of interwoven RNA features. The view, frequently referred to as the "crown view", looks like a halved pear with two lateral protuberances, called the L1 and L7/L12 stalks, is shown in Fig. (**1a**). Its flat surface faces the small subunit in the 70S ribosome and its round back side faces the solvent. The gross similarity of the rRNA folds of D50S to the available 50S structures, allowed superposition of D50S structure onto that of the 2.4 Å structure of H50S [16] and of the 50S subunit within the 5.5 Å structure of the T70S ribosome [23]. We found that the RNA fold and the overall protein distribution are rather similar in the three structures, but detected a few significant structural differences even within the conserved regions, which cannot be explained solely by expected phylogenetic variations.

Although the large subunit has less conformational variability than the small subunit, it does possess various conformations that can be correlated to the functional activity of the ribosome. The most significant differences between the two structures of the unbound large subunits were found in key features, known to participate in the functional activities of the ribosome. Remarkable examples are the 50S hook into the decoding region of the small subunit, and other intersubunit bridges created upon subunit association, the entire L1 arm [Fig. (**1a**)] that acts as the revolving gate for the exiting tRNA molecules, and the GTPase center. Most of these features are disordered in H50S [16] but well resolved in D50S [17]. Thus, D50S provides not only an excellent model for binding of antibiotics, but also the only crystal form that can lead to the design of new drugs that may target the flexible features and limit their mobility.

3.2.2. On the Formation of the Peptide Bond and the Exit Tunnel

The peptidyl transferase activity of the ribosome has been linked to a multi branched loop in the 23S secondary structure diagram, known as the peptidyl transferase ring (PTR). It is highly conserved among eubacteria. Among the 43 nucleotides forming the PTR 36 are conserved in *H. marismortui* and *D. radiodurans.* Superposition of the backbone of the high resolution structures of the PTR nucleotides in the two species [16] (and PDB 1JJ2) shows similar fold. The orientations of some of the nucleotides, however, show distinct differences. A2451 is the key element in the proposed peptide bond catalysis mechanism, based on the structure of H50S [1,16]. Biochemical evidence reveals the functional importance of U2504 and U2506. U2504 has been implicated in the binding of the 3'end of the aminoacyl tRNA prior to peptide bond formation [26,27] and U2506 was protected from chemical modification by P-site tRNA [28]. It is possible that the different orientations or locations of these bases reflect the flexibility needed for the formation of the peptide bond. It is also possible that the different orientations of these bases result from the differences in the functional states of the 50S subunit in the two crystal forms, since the structural changes occur at distinct nucleotides of the peptidyl transferase ring upon transition between the active and inactive conformations [5].

We recently showed that truncated substrate analogs bind to the ribosomal peptidyl center at a large range of conformations that may be similar, but not identical, to the mode of binding of larger RNA constructs that were designed to bind to the large subunit as tRNA mimics (Bashan *et al.*, to be published). These tRNA mimics include features representing the acceptor stem of tRNA, and were oriented by their interactions with two long ribosomal helical segments, H89 and H69. Such contacts cannot be created either for short analogs or when one of the main supports, helix H69, is disordered, as in the structure of H50S. Although this may not represent the actual translation of the genetic code, it is conceivable that some of the positions used by the short analogs may be suitable for the formation of single peptide bonds.

Analysis of the modes of attachment of the tRNA mimics to the peptidyl transferase center in D50S supports the idea that the ribosome provides a frame for the peptide bond formation, rather than being actively involved in the catalytic events, consistent with [3], and with the suggestion that di-metal ions may be instrumental for peptide bond formation [2]. Our studies also indicate that the peptidyl transferase center contains several flexible regions, some of which may be stabilized by the binding of substrate analogs.

More than three decades ago biochemical studies showed that the newest synthesized part of a nascent protein is masked by the ribosome [29,30]. In the mid eighties, a feature that may account for these observations was first seen as a narrow elongated region in images reconstructed from 80S ribosomes from chick embryos (at 60Å resolution) [31] and of 50S subunits of *Bacillus stearothermophilus* (at 45 Å) [32]. Despite the low resolution, these studies showed that this tunnel spans the large subunit from the location assumed to be the peptidyl transferase site to its lower part, and that it is about 100 Å in length and up to 25 Å in diameter [32], as confirmed later at high resolution in H50S [1] and in D50S [17].

4. ANTIBIOTICS TARGETING THE RIBOSOME

4.1. Antibiotics of the Small Subunit

As mentioned above, the most important task of the small subunit is to discriminate cognate from noncognate

tRNA molecules by monitoring base pairing between the codon of mRNA and the anticodon on the corresponding aminoacyl-tRNA (aa-tRNA). The decoding occurs at the A-site and requires the correct codon-anticodon recognition. Additional tasks are the participation in initiation and the termination of the entire process and in the translocation of the mRNA/tRNA complexes. All these activities provide a great variety of targets for antibiotics.

Already at the end of the eighties chemical probing of drug-ribosome complexes were used to show that many antibiotics interact with A-site-specific and P-site-specific bases in 16S ribosomal-RNA [33,34]. The current list of antibiotics interacting with the 16S RNA is rather large. It includes neomycin, paromomycin, gentamycin, kanamycin, apramycin, kasugamycin, myomycin, neamine, pactamycin, kasugamycin, tetracycline, edeine, streptomycin, spectinomycin, hygromycin and neomycin. Among these, gentamicin, kanamycin, paromomycin, apramycin, and neamine induce miscoding and inhibit translocation; kasugamycin inhibits fMet-tRNA binding; edeine, kasugamycin and pactamycin are known to interfere with initiation; streptomycin, tetracycline, spectinomycin, edeine, hygromycin and the neomycins protect concise sets of highly conserved nucleotides in 16S ribosomal RNA; and myomycin and streptomycin share similar modes of action.

The binding modes of the following antibiotics were investigated crystallographically: paromomycin and streptomycin, which interfere with decoding and translocation [10], spectinomycin, which hampers the head motion by trapping a particular conformation [10], streptomycin [10], hygromycin [11], and tetracycline, a multi-site antibiotic with inhibitory action that stems from its interference with A-site tRNA binding [11,12], pactamycin [11] and edeine [12]. Pactamycin and edeine are universal agents that inhibit mRNA binding by linking critical features translocation and E-site tRNA release, thus imposing constraints on ribosomal mobility required for the translation process.

4.1.1. Targeting the Decoding and Translocation

All the four aminoglycoside antibiotics, spectinomycin, streptomycin, paromomycin and hygromycin B that have been subjected to crystallographic studies bind near the decoding region. All function by altering delicate balances in the conformational states of the small subunit.

Spectinomycin acts on translocation, as shown earlier [35,36]. It inhibits the translocation of the peptidyl-tRNA from the A-site to the P-site, which apparently involves head movement. The structure of the spectinomycin/T30S complex shows that spectinomycin binds in the vicinity of the proposed pivot point, thus limiting head movement [10]. Interestingly, mutations that reduce translational fidelity are those that produce resistance to the antibiotic spectinomycin.

Streptomycin acts on the switch between the *ram* (ribosomal ambiguity mutation) and the restrictive states, and proteins S3 and S5 are involved in its binding. Streptomycin increases the proportion of error prone ribosomes by affecting the proof-reading step. In the complex with T30S it interacts with protein S5, suggesting that it preferentially stabilizes the *ram* state, thus increasing the initial binding of noncognate tRNAs [10].

Paromomycin and hygromycin B act on the decoding step. Paromomycin increases the error rate of the ribosome, by reducing the rate if the dissociation of A-site tRNA from the ribosome and increasing its binding affinity for tRNA [10]. Paromomycin binds at the decoding site and part of its structure is inserted into the RNA helix, thus assisting the flipping out of two bases involved in decoding. It also forms a tight hydrogen-bond interaction with the neighboring nucleotide to lock better the flipped-out bases.

Hygromycin B, which is a isolated from *Streptomyces hygroscopicus*, is an aminoglycoside with a single binding site close to the A-site, in a location known to move during translocation [37]. Its location is above the binding site for paromomycin in H44, as detected crystallographically [11] and suggested earlier [8,10]. In this location, the binding of hygromycin B can restrict the movement required for translocation.

4.1.2. Two Universal Antibiotics Limiting Platform Mobility and Interfering With P-Site Binding

The small subunit is the main player in initiation of protein biosynthesis. After binding to the mRNA the initiation complex moves in the 5' to 3' direction along the mRNA scanning it, in search for the initiator (AUG) codon [38]. As early as 1976 [39] it was found that edeine, aurintricarboxylic acid and polydextran sulphate block the binding of the mRNA to the small ribosomal subunit and that pactamycin induces the formation of stable smaller initiation complexes that are unable to go through the subsequent steps of initiation.

Edeine, kasugamycin and pactamycin, all universal antibiotics with no clinical relevance [40,41], are believed to affect translational initiation. They protect bases in common with P-site-bound tRNA. Kasugamycin enhances the reactivity of C795 and protects A794 and G926, whereas pactamycin protects G693 and C795. The major proteins photo-labeled using pactamycin derivates are S2, S4, S18 and S21 (in *E. coli*).

Edeine is a peptide-like compound, containing a spermidine-type moiety at its C-terminal end and a beta-tyrosine residue at its N-terminal end. It binds to the solvent side of the flatform [Fig. (**1b**)] in a position that may affect the binding of mRNA and the interaction of the 30S and 50S subunits [12]. It decreases the affinity constant of tRNAPhe for the P-site by more than two orders of magnitude, independently of the presence of poly(U). In addition, by physically linking the mRNA and four key helices that are critical for tRNA and mRNA binding, edeine locks the small subunit into a fixed configuration and hinders the conformational changes that accompany the translation process [42,43]. The binding of edeine to the 30S subunit induces the formation of a new base pair that may alter the mRNA path and would impose constraints on the mobility of the platform. Hence, it is conceivable that the

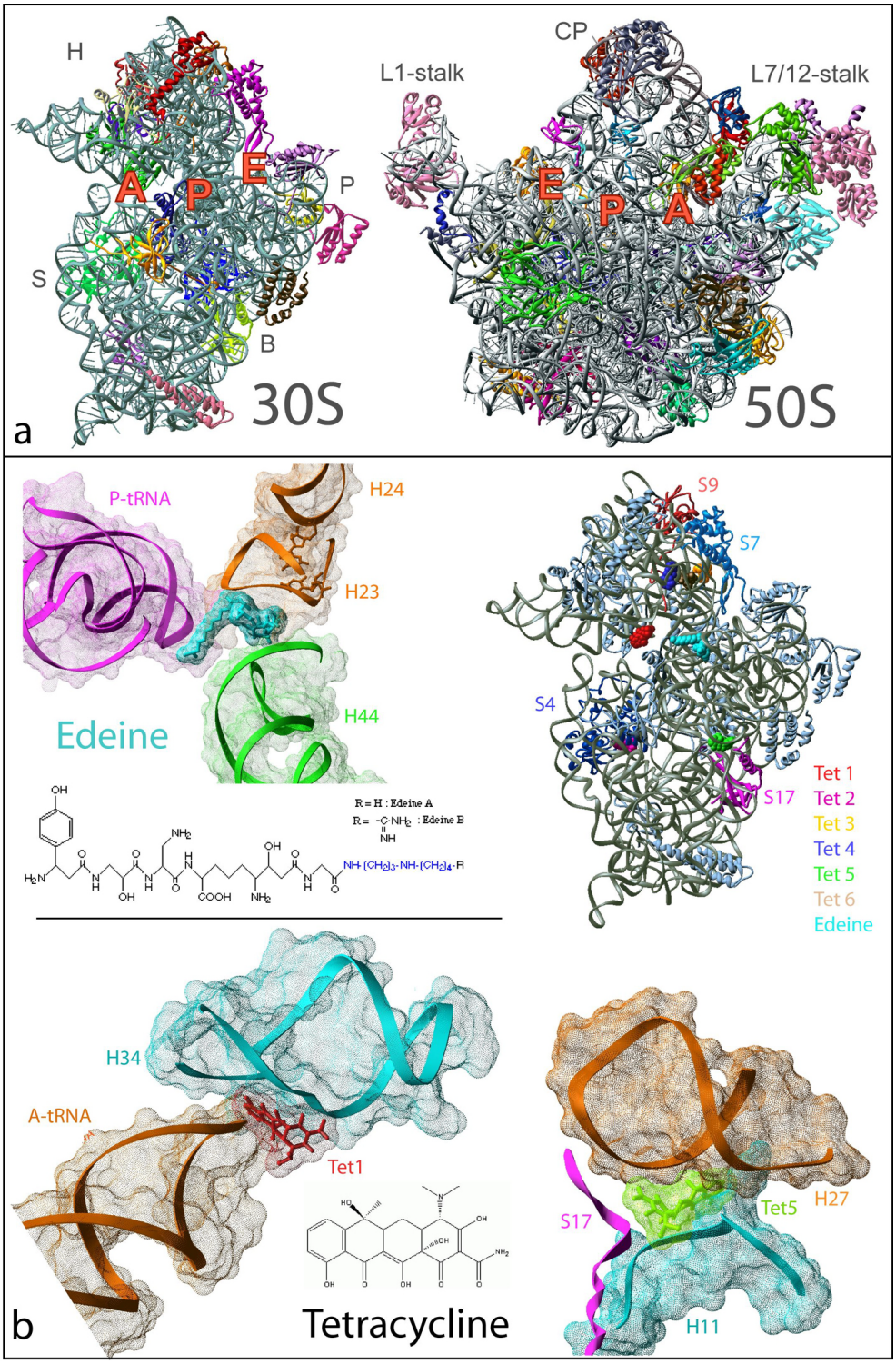

Fig. (1). (a) The high resolution structures of the two ribosomal subunits, as determined by us. The RNA chains are shown as ribbons and the proteins main chains in different colors. A, P and E designate the sites where the tRNA molecules interact with the small and the large subunits. Left: T30S [12]. H: head, B: body, P: platform, S: shoulder: Right: the crown view of D50S [17]with a circle designating the region including the peptidyl transferase center and the entrance to the tunnel. Both are shown from the side facing each other within the 70S ribosome. (b) The binding sites of edeine (top left) and of tetracycline (below). The positions of the binding sites of edeine and tetracycline within the entire subunit, are shown on the top right. The lower panel shows the two more relevant binding sites of tetracycline.

formation of this base pair interferes substantially with the initiation. Thus, our data suggest that the initiation process is the main target of this universal antibiotic, and that edeine induces an allosteric change by the formation of a new base pair-an important new principle of antibiotic action, that fits nicely with our hypothesis for the mechanism of the initiation step.

A subset of the 16S rRNA nucleotides protected by the P-site tRNA [44] overlaps with those of kasugamycin and pactamycin [34,45]. Edeine is called a P-site antibiotic since it disturbs P-site binding to the initiation complex while mRNA is being scanned. It also interferes with this process, so that the small subunit is unable to join the large one. Hence, it blocks peptide initiation and interferes with binding of methionyl-tRNAf to the small ribosomal subunits. Interestingly, the sets of nucleotides assigned as P-site protections are not essential for ribosomal function, and this probably has to do with the dynamics of the ribosome.

Pactamycin also interferes with the initiation process. It shares a protection pattern with edeine and bridges the same helices that are linked by the new base pair that is induced by edeine [45]. It is likely that besides reducing the mobility of the platform by locking these two helices, it also alters the mRNA path at the E-site. Like edeine, pactamycin interacts with the extended loop of protein S7, the upper border of the path of the exiting mRNA/tRNA complex, and its mode of interaction suggests that it may interfere with the pairing of the Shine-Dalgarno sequence or prevent it. Pactamycin belongs to the E-site antibiotics although it was described previously as a P-site agent. It interacts primarily with residues at the upper end of the platform and it displaces the mRNA [11].

The universal effect of edeine on initiation implies that the main structural elements important for the initiation process are conserved in all kingdoms. Analysis of our results show that the rRNA bases defining the edeine binding site are conserved in chloroplasts, mitochondria, and the three phylogenetic domains. Among these are two conserved nucleotides along the path of the messenger. Hence, an additional effect of edeine may be preventing hybridization of the incoming mRNA. Thus, both edeine and pactamycin show a novel mode of action, based on limiting the ribosomal mobility and/or preventing the ribosome from adopting conformations required for its function.

4.1.3. Tetracycline – A Multi-Site Antibiotic that Blocks A-Site Binding

The tetracyclines form a group of synthetic antibiotics acting against both gram-negative and gram-positive bacteria [46]. They are classified as “broad-spectrum” antibiotics [47], but due to rapidly developed resistance, they became less popular. Bacterial resistance to tetracycline is achieved by the presence of special enzymes that are either involved in exporting the drug across the cell membrane in an energy-dependent fashion (efflux), or that mimic the structure of the elongation factors. These proteins are able to approach the binding site and release the bound antibiotic from the ribosome [48].

Tetracycline has one primary and multiple secondary binding sites within the small subunit [49,50]. The relevance of the secondary binding sites remains unclear. In its primary binding site tetracyclines inhibit protein synthesis by interfering with the binding of aminoacylated tRNA to the A-site of the 30S subunit [7]. They are products of the aromatic polyketide biosynthetic pathways and belong to a family of bacteriostatic antibiotics that act against a wide variety of bacteria. Six tetracycline-binding sites, with no common structural trait and of different occupancies, were identified on the 30S subunit by us [12] [Fig. (**1b**)]. The observation of multiple tetracycline binding sites was not unexpected, since several binding sites for tetracycline were suggested based on biochemical experiments. The six positions revealed by us can well explain some of the contradictory reported biochemical and functional data for tetracycline binding to the 30S subunits [12].

The main tetracycline binding site lies in a clamp-like pocket formed by the head, at the A-site tRNA [Fig. (**1b**)]. Upon binding, the gap between the two bases forming the clamp becomes slightly wider. Aside from this local conformational change we did not observe any significant changes of the 30S subunit. The tetracycline in this site interacts with the sugar-phosphate backbone of H34 through a magnesium ion, in a fashion similar to the interaction between the Mg-tetracycline complex and the tetracycline repressor tet(R) [51]. The inhibitory action of tetracycline is mainly assigned to this site, where it interferes with the location of the anticodon loop of A-site tRNA. In this site, tetracycline can physically prevent the binding of the tRNA to the A-site. This mode of interaction is consistent with an independent crystallographic studies [11] and the classical model of tetracycline as an inhibitor of A-site occupation, and hence offers a clear explanation for the bacteriostatic effect of tetracycline.

Five additional binding sites for tetracycline were identified, most of them consistent with previous biochemical and functional studies [12]. The site that is most occupied is the one known to carry the antibiotic activity of tetracycline-preventing A-site tRNA binding. The second tetracycline binding site is located in a hydrophobic pocket of S4. This is the only tetracycline binding site not involved in interactions with the 16S rRNA. The third tetracycline binding site is buried inside a helix. The fourth site is located in a cavity at the bottom of the head. Site five lies in a rather tight pocket confined by H11, H20, H27, the switch helix, and protein S17, with its hydrophilic side interacting with the phosphate-sugar backbone of the switch region of H27, with the bulged H11. A similar, but not identical, binding site was seen by [11]. As mentioned above, helix 27 is known to be involved in a conformational switch that may be part of a larger-scale conformational change that occurs between initial selection and proof-reading of the A-site tRNA. The sixth site is located in the vicinity of the E-site, interacting with the N-terminal end of S7A, via a Mg^{2+} ion.

The presence of five additional binding sites, the biochemical evidence for different locations of tetracycline, and the low level of resistance conferred by the ribosomal

protection proteins, demand more complex explanations about the possible functional relevance of these additional sites. Although it is not certain that any of the five minor sites is involved in tetracycline action, we hypothesize that, with the exception of the tetracycline at the Tet-3 site, these sites could act synergistically to contribute to the bacteriostatic effect of tetracycline. The four proteins that contact tetracycline, S4, S7, S9 and S17 are primary rRNA binding proteins [52]. Therefore tetracycline binding at these sites could disturb the early assembly steps of the 30S particles, contributing to the overall inhibitory effect of tetracycline.

Tetracycline resistance is acquired by two ribosome-related mechanisms. Both relate to the Tet-1 site. In one, the resistance is mediated by ribosomal protection proteins that target the entire 70S ribosome, whereas in the other by the mutation on 16S rRNA [51,53,54]. Ribosomal protection proteins, such as TetM, TetO, and TetS, confer resistance only at low concentrations of tetracycline, and show some sequence and structural homology with the elongation factors G and EF-Tu. It has been proposed that TetM binds to the A-site and upon GTP hydrolysis actively releases the tetracycline bound to it. The mutation could hamper the local base pairing system and lead to a conformational change that results in closing the Tet-1 binding pocket. These two tetracycline resistance mechanisms reflect the importance of the Tet-1 binding site in the antibiotic action of tetracycline and indicate that the physical blockage of the A-site tRNA binding by tetracycline bound at Tet-1, can account for the inhibitory action of tetracycline.

4.2. Antibiotics of the Large Subunit

Antibiotics that bind to the large ribosomal subunit target three major regions. These include the ribosomal GTPase-associated center, which contains the binding sites for proteins L10, L12 and L11 and is inhibited by interaction with the antibiotic thiostrepton, the peptidyl transferase center, and the tunnel through which nascent proteins progress until they emerge out of the ribosome.

Among the less expected, albeit reasonable, antibiotics binding sites is that of the antimicrobial agents evernimicin, an oligosaccharide antibiotic that inhibits bacterial protein synthesis. It interact with the large ribosomal subunit at the hairpins of helices 89 and 91 of the 23S rRNA, based on spontaneous resistant mutants of *Halobacterium halobium*, in the vicinity of the archaeal protein L10e, which is homologous to protein L16, the protein that makes several interactions with the vicinity of A-site tRNA. The locations of these mutations overlap with the binding site of initiation factor 2, and were shown to inhibit the activity of initiation factor 2 *in vitro* [55].

4.2.1. The GTPase-Associated Center

GTP hydrolysis provides the energy that allows the protein biosynthetic process to proceed against a thermodynamic gradient. Two thio-peptide antibiotics, thiostrepton and micrococcin, bind to protein L11 and inhibit growth of the pathogen-model bacteria, such as *E. coli* and *Bacillus stearothermopilus* [56,57], as well as malaria parasite *Plasmodium falciparum* [58-60].

Thiostrepton inhibits functional transition within protein L11 [61] and the overall turnover but not the GTPase of elongation factor G on the ribosome [62]. Protein L11 together with the 23S rRNA stretch that binds it, are associated with elongation factor and GTPase activities [56]. This highly conserved region is the target for the antibiotic thiostrepton and micrococcin, and that cells acquire resistance to this antibiotic by deleting protein L11 from their ribosomes. Thus, large ribosomal subunits lacking protein L11 cease to bind thiostrepton and micrococcin but do not undergo major conformational changes [57,63]. Furthermore, ribosomes from the "L11-minus" strains crystallized under the same conditions and formed crystals of the same symmetry and identical unit cell dimensions as of the wild-type large ribosomal subunits. A few years ago a new class of thiostrepton-resistant mutants of the archaeon *Halobacterium halobium* were identified and found to carry a missense mutation in the gene encoding ribosomal protein L11. The mutant protein carries either serine or threonine instead of a proline that is conserved in archaea and in eubacteria and these mutations do not impair the assembly of the 70 S ribosomes *in vivo.* A similar mutant for micrococcin has been identified in *Bacillus megaterium,* in which insertions or nonsense mutations of L11 led to a slow growth of the cells.

Complexes containing L11 or one of its domains together with fragments mimicking the RNA stretch binding it, were subjected to crystallographic and NMR studies [64,65]. Interestingly, whereas the structures of protein L11 determined in these studies are similar to that found within the ribosomal particle, there is less resemblance between the structures of the RNA fragments and that determined *in situ*.

4.2.2. The Macrolides Bind to the Entrance of the Exit Tunnel

Macrolides are clinically important antibiotics. They, and their newer derivatives, the ketolides, act against gram-positive aerobes and some gram-negative aerobes. Most macrolides have a broad-spectrum antimicrobial activity and are used primarily for respiratory, skin and soft tissue infections.

The macrolide family is large and structurally diverse. The central component of the macrolides, a lactone ring carrying a number of substitutions, is shown in Fig. (**2**). This macrolactone ring is composed of a polyketide core and contains from 12 to 16 atoms, to which one or more sugars, usually neutral or aminodeoxysugars in the form of mono- or disaccharides are attached via glycosidic bonds. Erythromycin A was the first to be used for therapy almost 50 years ago. It belongs to the first generation of 14 member ring macrolides. Erythromycin, oleandomycin, spiramycin, josamycin and midecamycin are natural compounds. All have 14 member rings. Erythromycin is produced by the bacterium *Saccharopolyspora erythrae*a, and has been extensively modified chemically (see below). Recently the way to its genetic modification was facilitated since the three dimensional structure of the macrocycle-forming thioesterase

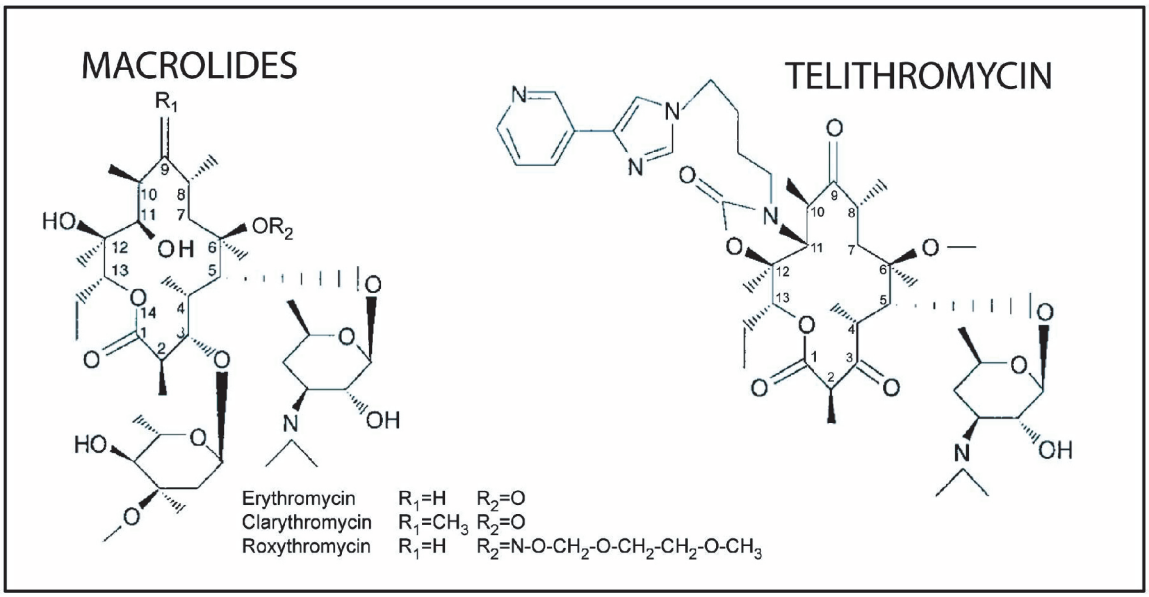

Fig. (2). The general formula of a 14-member macrolide and the positions that had been chemically modified.

domain of the erythromycin polyketide synthase has been determined [66].

Semi-synthetic molecules differ from the original compounds in the lactone ring, which is substituted by hydroxyl or alkyl or ketone at C7 in 12-membered macrolides and at C9 in 14-membered macrolides, and an aldehyde group in 16-membered macrolides. In recent years numerous new 14-membered macrolide derivatives of erythromycin A were synthesized and found to be more stable and have a broader spectrum of action due to chemical modifications of a hydroxyl group at C6, a proton at C8, or a ketone at C9. Synthetic derivatives, such as dirithromycin, roxithromycin, clarithromycin and flurithromycin, have increased stability under acidic conditions, and chlarithromycin was found to have clinically useful activity against atypical mycobacteria. Also, the 15-membered macrolide, azithromycin, with a methylated nitrogen inserted into the lactone ring shows good activity against Gram-negative bacteria [67,68].

The 14-member ring macrolides, shown in Fig. (**2**) are among the most important antibiotics due to improved stability and spectrum. Macrolides of the second generation, such as clarithromycin or roxithromycin are used extensively in clinical settings. Subsequent rapid spread of antibiotic-resistant strains has stimulated the search for additional novel derivatives. The macrolides of the third generation are called ketolides, since they contain a keto group instead of the cladinose residue at position 3 of the lactone ring and carry alkyl-aryl side chains. The ketolides not only show an improved activity profile against sensitive bacteria, but also are more active against certain macrolide-resistant strains.

Macrolides bind reversibly to the 23S rRNA, and the main components that interact with them have been identified almost two decades ago [69]. They do not inhibit the catalytic activity of ribosomal peptidyl transferase, but rather interfere with the release of nascent proteins, and thus inhibit protein synthesis by blocking the entrance of the protein exit tunnel. Most of them do not affect the peptidyl transferase activity [70] and do not share binding sites with the peptidyl transferase inhibitors, such as chloramphenicol.

Macrolides were shown to compete with peptidyl tRNA for binding to the ribosome and therefore lead to spontaneous dissociation of the peptidyl-tRNA before entering the exit tunnel. This property may explain results from earlier studies that indicated partial competition between the chloramphenicol and erythromycin [71]. In recent years, the 16-membered macrolides carbomycin, tylosin, and spiramycin were shown to exhibit some interactions to the peptidyl transferase center and to inhibit some peptidyl transferase activity [72].

The structural bases for the interactions of three macrolides antibiotics, erythromycin, chlarithromycin and roxithromycin have been elucidated [Fig. (**3a-d**)]. They sterically hinder the progression of the nascent proteins through the exit tunnel, and their binding sites are composed exclusively of segments of 23S rRNA at the peptidyl transferase cavity [13]. The high affinity of the macrolides (K_{diss} 10^{-8} M) to the ribosome is difficult to explain solely by their hydrogen bonding scheme. Hence it is likely that their binding is being further stabilized by van der Waals forces, hydrophobic interactions, and the geometry of the rRNA that tightly surrounds the macrolide molecules.

All three macrolides bind to the 23S RNA at the entrance of the protein exit tunnel, consistent with previous suggestions that they to block the progression of the nascent peptide [73,74] and stimulate the dissociation of peptidyl-

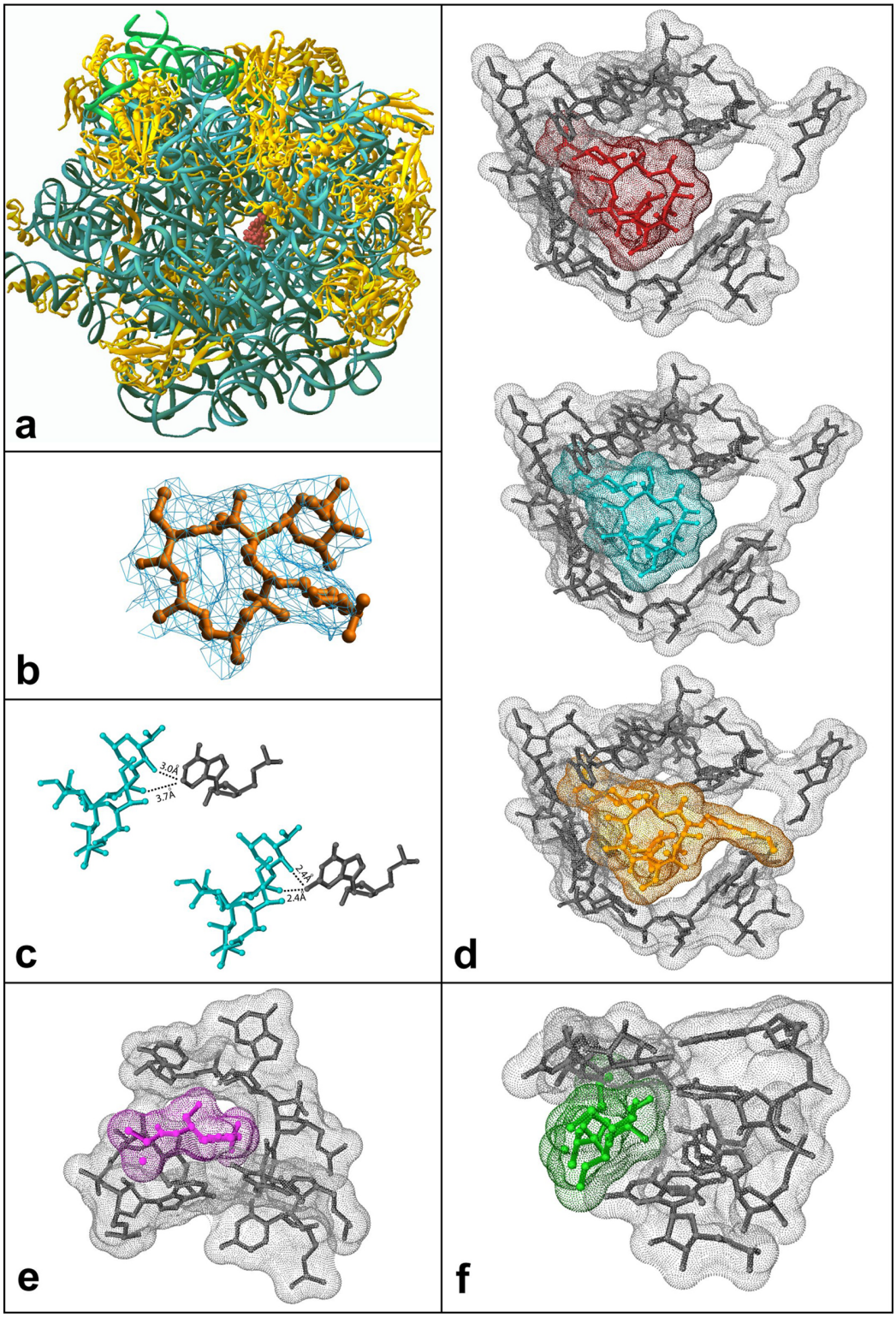

Fig. (3). (a) to (d): Binding of macrolides. (a) Top left: The position of erythromycin (red) within the RNA components at tunnel entrance in large subunit from *D. radiodurans* (in yellow-green). (b) A typical difference electron density difference map of a macrolides bound to the ribosome. (c) Top-left: the environment of A2058 (in gray) and erythromycin. Bottom-right: the same as on top-left, but the Guanine was placed on the Adenine. (d) Semi-space-filling models of erythromycin (red), chlarithromycin (cyan), and roxithromycin (gold), placed at the entrance to the tunnel. The walls of the tunnel are shown in gray. (e and f) Chloramphenicol and clindamycin, respectively, shown within their binding pockets as semi-space filling models.

tRNA from the ribosome [75]. The binding site of erythromycin allows the formation of 6-8 peptide bonds before the nascent protein chain reaches the blocked entrance to the tunnel, as suggested earlier [76]. Moreover, in order to reach this narrow passage the nascent peptide needs to progress in a diagonal direction, thus imposing further limitations on the growing protein chain. Once macrolides are bound, they reduce the diameter of the tunnel from the original 18-19 Å to less than 10 Å, and, since the space not occupied by erythromycin hosts a hydrated Mg^{2+} ion, the passage available for the nascent protein is 6-7 Å.

As in the small ribosomal subunit, ribosomal proteins may affect the binding and action of ribosome-targeted antibiotics, but the primary target of these antibiotics is rRNA. The two ribosomal proteins that have been implicated in erythromycin resistance are L4 and L22. However, the closest distances of erythromycin to these proteins are 8-9 Å, which are too long for meaningful chemical interactions. Therefore it was suggested that the macrolide resistance acquired by mutations in these two proteins is an indirect effect, produced by a perturbation of the 23S rRNA induced by the mutated proteins. These perturbations may or may not be connected to the changes in the width of the protein exit tunnel, a proposal based on cryo electron microscopy studies performed at low resolution [77].

The binding mode of the macrolides may assist in understanding antibiotic selectivity. Among them, erythromycin is known to bind weakly to eukaryotic ribosomes, as do other members of the 14-mer macrolides. Among other interactions [13], this antibiotic interacts with A2508, a nucleotide that confers erythromycin resistance and reduced affinity for other macrolides when methylated by Erm-type methyl-transferases. [71,78]. In eukaryotes and several archaea, e.g. *H. marismortui*, this base is a guanine instead of adenine. It was found that this A to G replacement decreases the affinity of the antibiotic to the archea and eukaryotic ribosomes, thus limiting the binding of 14-member macrolides to the large subunit. It is therefore not surprising that binding of erythromycin to H50S required over 1000 fold higher concentration than that needed for binding to D50S, E50S or typical pathogenic bacteria, and that the orientation of the H50S bound erythromycin is tilted [79] compared to that bound to D50S [13].

To shed light on this point, we substituted (computationally) the adenine in position 2058 (*E. coli* numbering) by a guanine, within the D50S structure. Adenine2508 forms an extensive set of interactions with erythromycin [13]. Fig. (**3c**) shows that guanine in position 2058 makes the accommodation of erythromycin is virtually impossible, assuming that erythromycin should bind with the same orientation found by us for all the D50S bound 14-, 15- and 16 member macrolides (Schluenzen *et al.*, to be published). In support of our structural results, mutation of G2058 to adenine in the large subunit from *H. marismortui* significantly increased their affinity for erythromycin [80].

Ketolides are 14-membered macrolides with a 3-keto in place of the L-cladinose moiety. The ketolides were reported to bind to 23S rRNA and their mechanism of action is similar to that of macrolides. Ketolides are active on macrolide-resistant species, such as Streptococcus spp, including mefA and ermB producing *Streptococcus pneumoniae.* The ketolides exhibit an improved activity profile and show significant activity against some macrolide-resistant isolates. Specifically, the ketolides, as the newer macrolides, azithromycin and clarithromycin, show higher stability and improved activity against *Haemophilus influenzae*, compared to erythromycin [81]. Telithromycin is the first to become use clinically, and represents a new generation of antimicrobials. Its structure was derived from macrolides, to which several features, important for its improved microbiological profile, were added [82]. The increased ribosomal affinity of telithromycin correlates with its superior potency against gram-positive *cocci* both *in vitro and in vivo*.

4.2.3. Antibiotics at the Peptidyl Transferase Center

The peptidyl transferase center within the large subunit serves as the major binding target for many antibiotics, including substrate analogs such as puromycin [73]. Antibiotics that arrest protein synthesis are, for example, chloramphenicol, lincosamides (e.g. clindamycin) and the oxazolidinones.

Both chloramphenicol and clindamycin inhibit peptidyl transferase by interfering with the proper positioning or with the translocation of the tRNAs at the peptidyl transferase cavity [Fig. (**3e and f**)]. Chloramphenicol targets the peptidyl transferase cavity close to the amino acceptor group of tRNA. Clindamycin interferes with substrate binding and physically hinders the path of the growing peptide chain. Interestingly, neither of these antibiotics binds to the nucleotides assigned to be crucial for the catalytic mechanism of the ribosome that was proposed based on the 2.4 Å structure of the *H. marismortui* large subunit [1].

Chloramphenicol is similar to the oxazolidinones in inserting mistakes in translation. It blocks peptidyl transferase activity by hampering the binding of tRNA to the A-site [33,73]. Chloramphenicol targets mainly the A-site. It interferes with the aminoacyl moiety of the A-site tRNA. In the crystal structure of the complex of chloramphenicol with D50S [13], one of its reactive oxygens forms hydrogen bonds with a nucleotide (C2452), which was previously shown to be involved in chloramphenicol resistance. The binding site of the lincosamide clindamycin partially overlaps with that of chloramphenicol.

Lincosamide antibiotics, like the macrolides, stimulate peptidyl-tRNA dissociation from the ribosome [83]. These antibiotics allow the synthesis of small peptides, which dissociate as peptidyl-tRNAs before being completed. Clindamycin is a semi-synthetic agent that acts as a bactericide in the treatment of primarily infections involving anaerobes and gram-positive cocci, problems in some clinical settings. It interacts with the A- and P-sites [84], thus sharing some properties with the macrolides.

Crystallographic studies showed that clindamycin interferes with the A-site and P-site substrate binding and physically hinders the path of the growing peptide chain

[13]. In this way it bridges between the binding site of chloramphenicol and that of the macrolides. It overlaps with both A- and P-sites, explaining it's A/P hybrid nature and links regions known to be essential for the proper positioning of the aminoacyl- and peptidyl-tRNAs and thus limits the conformational flexibility needed for protein biosynthesis. These overlapping binding sites explain why clindamycin and macrolides bind competitively to the ribosome and why most RNA mutations conferring resistance to macrolides also confer resistance to lincosamides.

Linezolid belongs to the synthetic antibiotics oxazolidinone family that inhibits protein synthesis in gram positive bacteria, including the halophilic archaeon *Halobacterium halobium* [85]. Oxazolidinones are active against multidrug-resistant gram-positive bacteria [86]. They do not show cross-resistance with other known inhibitors of translation, but binding of oxazolidinones to the large ribosomal subunit is inhibited by chloramphenicol, lincomycin and clindamycin, that were shown to bind to the ribosomal peptidyl transferase center [13].

5. ON RESISTANCE, SELECTIVITY AND UNIVERSALITY

Resistance to antibiotics creates major problem in modern therapeutics. Drugs that gained popularity and have been used extensively as bactericidal agents in human and veterinary medicine, select for the emergence of dominant mutations in clinically important pathogens that respond to the pressure of antibiotics. These bacteria develop sophisticated defense mechanisms to combat the damage caused by the antibiotics to their metabolism. Resistance to one family may confer resistance to members of other families. For example, in the US, up to 25% of *Streptococcus pneumoniae* strains were found to be resistant to macrolides and more than 50% of the *S. pneumoniae* strains that were resistant to penicillin were also resistant to macrolides.

MLS is a resistance superfamily that includes chemically distinct, but functionally overlapping antibiotics. It includes the macrolide, lincosamide and streptogramin B families. The macrolides include carbomycin, clarithromycin, erythromycin, josamycin, midecamycin, mycinamicin, niddamycin, rosaramicin, roxithromycin, spiramycin, and tylosin. Celesticetin, clindamycin, and lincomycin belong to the lincosamide family, and staphylomycin S, streptogramin B and vernamycin B belong to the streptogramins family. Members of these three families interact competitively with the 50S subunit, and only one antibiotic molecule at a time can bind to a single 50S subunit.

Resistant strains may persist for long periods. Thus, there is great interest in the development of improved antibiotics alongside new antibiotic agents to circumvent existing patterns of resistance. Thus, the clinical use of several powerful agents has been limited by the emergence of widespread strains of bacteria with resistance to these drugs.

Bacterial resistance is either inherent or acquired after exposure to a specific antibiotic agent. Inherent resistance is meant also to protect the producing organism from its own antibiotic compound and may involve some mechanism for removal of the antibiotics. Acquired resistance may result from a mutation that will be passed on to the next generations or from the introduction of transferred genetic material from other microorganisms or viruses.

The molecular mechanisms whereby bacteria become antibiotic resistant usually involve drug efflux, drug inactivation, mutations designed to alter the target site in order to impair the binding of the antibiotics, or the production of enzymes that inactivate the antibiotic (e.g. beta-lactamase). In addition, enzymes that modify the target site post-translationally are produced, for example enzymes that acetylate, phosphorylate or adenylylate the target. Changes in cell membrane permeability hamper the entrance of antibiotics and efflux pumps are used in many cases to remove the antibiotics. Resistance can also be achieved by bypassing critical steps in the target pathway (e.g. for sulfonamide and trimethoprim) or by the synthesis of special proteins that are able to remove the antibiotics (e.g. tetracycline, see above for more detail). Interestingly, mechanisms involving mutational alteration of genes that normally reside in the host and that encode either ribosomal protein or rRNA have also been described.

An important reason for resistance stems from self-defense of the producing organisms. Thus, organisms that produce aminoglycosides protect themselves by a methylase that specifically modifies 16S rRNA nucleotides (e.g. kanamycin, gentamycin, apramycin, and istamycin). Similar argumentation holds for universal antibiotics. An example is edeine [40]. All the rRNA bases defining the edeine-binding site are conserved in chloroplasts, mitochondria, and the three phylogenetic domains [12]. As all rRNA bases involved in edeine binding are also conserved in *Brevibacillus brevis*, the organism that synthesizes it, *B. brevis* rapidly releases the active edeine into the growth medium, or maintains a low concentrations of inactive edeine attached to the internal part of the cell membrane [87].

The antibiotics binding modes that have been identified recently by crystallographic methods [10-13] are consistent with antibiotic resistance linked to alterations of specific nucleotides of the ribosomal RNA [88]. These show that the primary target is the ribosomal RNA, although ribosomal proteins may affect the binding and action of ribosome-targeted antibiotics.

The main mechanism of macrolide resistance is based on a modification of the drug binding site. For example, it was found that the great majority of the macrolide-resistant streptococci and staphylococci have become resistant through the acquisition of a gene designated *erm* (erythromycin ribosome methylation). This gene encodes an N-methyltransferase that methylates the N-6 amino group of A2058 in 23S rRNA, a modification that reduces the binding of the macrolides by over 1000-fold [71]. This adenine-specific *N*-methyltransferase acts at the

posttranscriptional stage and can confer resistance not only to most of the macrolides, but also lincosamides and streptogramin B [89].

Almost three dozens of erm genes have been isolated and characterized from bacterial sources, including clinical pathogens. A tabulation of the *erm* genes was published within a detailed review on the resistance to erythromycin by ribosome modification [71]. Furthermore, although the 16-membered macrolides are relatively poor inducers of resistance, nevertheless, an *ermC* was identified in staphylococci isolated from animals that received tylosin.

Resistance to antibiotics can also occur by chemical modification, for example phosphorylation and glycosylations (see Smith and Baker in this issues). For macrolides, lactone ring cleavage by erythromycin esterase has been detected and modifications in the 23S rRNA within the peptidyl transferase center were reported [78]. Similarly, single-point mutations in 23 S rRNA confer resistance to linezolid. Active efflux is another mean for resistance to erythromycin and streptogramin B [73] but does not affect clindamycin.

Mutations conferring resistance to antibiotic inhibitors of translation occur also in ribosomal proteins, suggesting that some antibiotics might act by interfering with functions mediated by ribosomal protein-rRNA interactions. As mentioned above, thiostrepton resistance can be achieved by the deletion of protein L11. Mutations to erythromycin resistance that involve alterations in ribosomal proteins in *E. coli* [90], *Bacillus subtilis* [91] and *Bacillus stearothermophilus* [92] were reported almost 30 years ago. Recent studies indicate a deletion of three amino acid residues (Met82, Lys83 and Arg84) in protein L22, and a single amino acid substitution, Lys63 to Glu in protein L4 lead to erythromycin resistance [93]. Altered protein S5 was found in spectinomycin-resistant mutants like *spc-13* [94]. Alterations in protein S17 [95], protein L6 [96] have also been detected. Altered protein S12 was detected in streptomycin-resistant mutants like *strA* [97], and L12 affects the reactivity of nucleotides in 16 S rRNA to chemical probes [98]**.**

Even in early days it was suggested that the amino acid alterations found in L22 mutants play only an indirect role in erythromycin resistance and action. Hence, it was proposed that the mutations in ribosomal proteins L22 and L4 perturb the structure of the 23S ribosomal RNA [93], an idea that has been partially confirmed at low resolution by cryo-electron microscopy [77] and it remains to be seen at higher resolution, once crystals of such mutants are obtained. However, erythromycin may inhibit the assembly of the large subunit if bound to proteins L22 and L4 prior to the initiation of this process, since these two proteins form *in vitro* an early assembly intermediate [99,100]consistent with biochemical studies indicating that erythromycin and azithromycin interfere with ribosome assembly [101].

Additional studies indicated that 16-membered ring macrolides, such as carbomycin, niddamycin, and tylosin bind selectively to protein L27, whereas erythromycin, a 14-membered ring macrolide, does not. These observations are consistent with studies that show that L27 was crosslinked to RNA bases that were located in the high resolution structures of the 50S subunit in the vicinity of the peptidyl-transferase cavity [102,103]. The 16-member macrolides are not only larger but also have longer chemical “arms” than those of the 14 members macrolides. Therefore, in principles, they can reach out from their main binding site, at the entrance to the protein exit tunnel, towards the peptidyl-transferase center. A rearrangement of the flexible N-terminal end of protein L27, which is consistent with the positioning of this protein in high resolution structure of the unbound subunit of D50S [17], is required to justify L27/16-macrolide interactions as well as for the crosslinking reported for protein L27 and the vicinity of the ribosome active site.

Recently it was found that short peptides can act as regulatory nascent peptides and render resistance to macrolides [71] [Lovett, 1996 #143 [104-106], while exploiting the peptides translated by the same ribosome. The length and the sequence of the peptides are critical for their activity, suggesting direct interaction between the peptide and the drug on the ribosome. Some interplay has been detected *in vivo* between nascent peptides stalling the ribosome and low concentrations of the antibiotics chloramphenicol, erythromycin and several ketolides in the Ribosomal Tunnel. It remain to be seen if this interplay is a consequence of rearrangements of the structure of the accumulating mRNA once the ribosome is stalled, or it is mediated by the very short nascent peptides, which might escape from the ribosome before they enter the tunnel.

To further verify the suitability of *D. radiodurans* to serve as a pathogen model, we attempted to isolate spontaneous mutants. Universal as well as specific antibiotics were used, including clindamycin and various 14- and 16-member macrolides. Seven such mutants were obtained, and their doubling time increased from the normal 3 to about 6 hours. All such obtained mutants were checked for cross resistance with other antibiotics. No cell growth occurred using 30S antibiotics or chloramphenicol, but mutants resistant to one macrolide could grow on media containing another macrolide. Preliminary observations indicated that most of these mutants are indeed at the ribosome level, and the design of oligomers that should pair with the regions expected mutated region is underway.

Surprisingly, one of the so obtained spontaneous mutants has 70S ribosomes with increased life time and less tendency to dissociate. Fig. (**4**) shows sucrose gradient runs (15-30%) of purified wild-type and mutated 70S after one or five days incubation, respectively. As seen, the native 70S, dissociated partly into 50S and 30S subunits whereas the mutant shows one stable peak even after the incubation conditions that led to the dissociation of the native ribosome. This may indicate that the binding site of this drug is in the vicinity of one of the intersubunit bridges [23], presumably near the bridge combining the peptidyl-transferase cavity in the large subunit and the decoding region in the small subunit [17]. Resistance to this drug may be achieved by hampering subunit dissociation, thereby limiting the pool of free subunits, or by restricting the inter-correlated intersubunit movements during translocation.

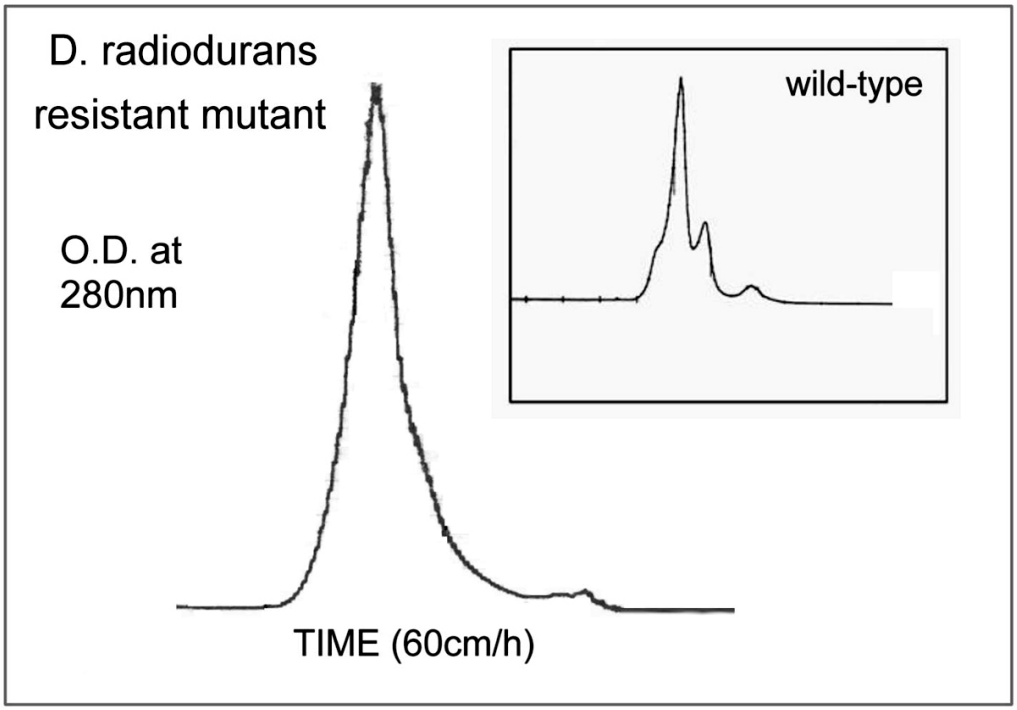

Fig. (4). the stability of the mutated ribosome from *D. radiodurans*. The main figure shows 70S ribosomes seven days after preparation. Wild-type ribosomes, 48 hours after preparation, are shown in the lnsert. Both preparations were obtained as described below and kept under the same conditions, except for 10 mM Mg^{++} used in the wild-type solution and only 3 mM in the solution of the resistant-mutant. Antibiotic resistant mutants were obtained by selective growth on plates containing 40-75 µg/ml of the antibiotic. The plates were incubated at 30°C and colonies appeared after four days. The spontaneous mutants were picked and inoculated into medium containing 75-150 µg/ml of the antibiotic. After three days of incubation the mutants were spread on plates containing 170-200 µg/ml of the antibiotic. In control experiments no growth of wild-type cells was observed. Also, no cross correlation with chloramphenicol was detected.

Selectivity between eubacterial and eukaryotic targets is one of the main properties that dictate the usefulness of drugs. Some structural basis for the elucidating of general aspects concerning selectivity is already available, since comparative studies illuminated some elements that may confer drug selectivity. An example is erythromycin, which like most of the 14-member macrolides, done not bind to eukaryotic ribosomes. Halophilic bacteria, being archea, have dual properties. They behave in some respects as eubacteria and in others as eukaryotes. Thus, comparative studies between the Achaean *H. marismortui* and the eubacteria *D. radiodurans*, may illuminate elements that may confer drug selectivity. As mentioned above, the nucleotide at position 2058 was identified as the base for the selectivity of the fourteen member rings macrolides as well as of lincomycin. As seen in Fig. (**3**)**,** the differences in the mode of binding of erythromycin in D50S and in H50S may reflect the differences between eubacteria and eukaryotes, since the pathogenic typical A2058 is G2058 in *H. marismortui* as well as eukaryotes. Importantly, one of the members of the halophilic family, *Halobacterium halobium* was extremely helpful in understanding antibiotics activity and resistance. This strain possesses a single chromosomal copy of the rRNA operon, hence allowing the direct isolation of resistance mutations [19].

Clindamycin may shed light on a different aspect of antibiotic selectivity. Its selectivity is connected not only to 2058, similar to the macrolides, but also to position 2059. It makes extensive contacts with the eubacterial ribosome, and when modeled into the active site of *H. marismortui*, it was found that it can forms only a few interactions. Clindamycin occupies parts of the binding sites of chloramphenicol and the macrolides, and hence shares properties with both of them. Interestingly, we found that the quality of the co-crystals of clindamycin with D50S is higher than of the native ones, probably because this antibiotic limits the flexibility of the region between the active site and the exit tunnel.

Puromycin, which was named an Òantibiotic agentÓ since it is a product of a microorganism, is a universal ribosome

several cases tumors cells are more permeable to universal antibiotics than the normal ones, allowing the use of these antibiotics as antitumor agents with reduced effectiveness in healthy cells. An example is the pleuromutilin-derived semi-synthetic antibiotic tiamulin, that at low concentrations sensitizes the highly resistant overexpressing tumor cell lines of murine lymphoid leukemia, rat hepatoma and human lymphoblastic leukemia [111]. Thus, the contribution of the universal antibiotics in therapeutics is not negligible.

6. CONCLUSIONS

Ribosomes are major targets for antibiotics. Ribosomal crystallography, initiated two decades ago, has recently yielded exciting structural and clinical information as antibiotics targeting ribosomes were found to be excellent tools for studying ribosomal function and for understanding mechanisms of drug action. The structural studies shed light on only on antibiotics actions on ribosomes but also on the mechanisms conferring resistance to antibiotics. Resistance to antibiotics is a major problem in modern therapeutics. With the increased use of antibiotics to treat bacterial infections, pathogenic strains have acquired antibiotic resistance, and thus antibiotics are becoming less effective. Antibacterial resistance is becoming an extremely serious medical problems that has prompted extensive effort in the design of modified or new antibacterial agents.

The newly determined structures of the ribosome and its complexes with antibiotics constitute powerful tools for understanding the modes of action of the antibiotics as well as the mechanisms conferring drug resistance, and thus should be useful for rational design and for the selection of novel and/or enhanced antibiotics. Since the antibiotics bind in locations that are crucial for the ribosomal functions, the emerging structural data could also be used to elucidate how the ribosome performs its crucial biological role in the translation of the genetic code.

Appendix 1. Glimpse into the antibiotics' diversity

Aminoglycosides are bactericidal antibiotics. They bind irreversibly to the small ribosomal subunit and interfere mainly in the decoding and translocation steps. They are effective in the treatment of severe infections caused by gram-negative bacteria, including the Pseudomonas species.

Macrolides inhibit the emergence of the nascent proteins. They are active against gram-negative anaerobes and most gram-positive cocci, but not enterococci. Erythromycin, the earliest natural product belonging to this group, and is produced by a mould Streptomyces. Many semi-synthetic derivatives with prolonged half-life in the body have been produced and found to be suitable for short-term therapy. Macrolides are the efficient antibacterial agents in treatment of typical as well as atypical pathogens (e.g. Mycoplasma, Chlamydia and Legionella).

Tetracyclines are broad-spectrum synthetic antibiotics that can act as bactericidal as well as bacteriostatic agents, depending on the dose. They enter the bacterial cell by an energy driven process, bind reversibly to the small ribosomal subunit. They inhibit protein biosynthesis by preventing the binding of the aminoacyl tRNA to the A-site. Tetracycline was modified to produce either short or long acting agents. All penetrate tissues well and are effective not only against gram-positive and gram-negative bacteria, but also for treatment of mycoplasmas, chlamydiae and rickettsiae. Successes in treatment of various infectious diseases, including gastrointestinal infections by Salmonella, *Vibrio cholerae* and Campylobacter, led to the intensive use of tetracyclines in human and veterinary medicine, and led to extensive resistance of many bacteria.

Sulfonamides have been in use as effective antimicrobial agents for over seventy years. They interfere with the folic acid pathway, thus inhibiting DNA synthesis in a wide spectrum of gram-positive and gram-negative bacteria, toxoplasmas and plasmodia. Being longest in use, many bacterial species have developed resistance to sulfonamides, and hence they have been modified and improved.

Beta-lactams (including penicillins, cephalosporins, carbapenems, and various beta-lactamase-inhibitors, such as clavulanic acid or sulbactam). The penicillins disrupt the bacterial cell wall by inhibiting the enzymatic synthesis of peptidoglycan, an essential cell wall component. Cephalosporins, developed to combat penicillin-resistant infections, bind to penicillin binding proteins and inhibit the synthesis of the bacterial cell wall in gram-positive and gram-negative bacteria. Beta-lactamase is the bacterial enzyme leads to acquired resistance to the penicillins and Cephalosporins, and beta-lactamase-inhibitors were developed against it. Glycopeptide antibiotics (e.g. vancomycin) were designed to overcome penicillin resistant staphylococci by binding to a cell wall precursor, thus inhibiting peptidoglycan synthesis. They are active against aerobic and anaerobic gram-positive bacteria.

Fluoroquinolones are broad-spectrum, potent synthetic bactericidal antibiotics, inhibiting DNA gyrase, an enzyme essential for DNA replication, recombination and repair. They act against enterobacteriaceae that have acquired resistance to many classes of antibiotics and some naturally resistant pathogens, such as Pseudomonas. They are active against most gram-positive bacteria, strictly anaerobic and atypical' pathogens such as Chlamydia, Mycoplasma, and Legionella.

Others. Antibiotics that target ribosomes and do not fall into any of the above categories are either clinically relevant (e.g. chloramphenicol, the lincosamides and the oxazolidinones), or are universal (e.g. edeine, pactamycin, puromycin and sparsomycin). The universal antibiotics are not useful in therapy, but may indicate novel designs for potent drugs.

ACKNOWLEDGEMENTS

We thank, M. Wilchek, A. Tocilj, R. Wimmer and W. Traub for critical discussions and R. Albrecht, W.S. Bennett, H. Burmeister, C. Glotz, G. Goeltz, H.A.S. Hansen, M. Laschever, M. Peretz, C. Radzwill, B. Schmidt,

A. Sitka and A. Vieweger for contributing to different stages of these studies. These studies could not be performed without the cooperation of the staff of the synchrotron radiation facilities at EMBL & MPG beam lines at DESY; ID14/2 & ID14/4 at EMBL and ESRF and ID19/APS/ANL. Support was provided by the Max-Planck Society, the US National Institute of Health (GM34360), the German Ministry for Science and Technology (Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie Grant 05-641EA), and the Kimmelman Center for Macromolecular Assembly at the Weizmann Institute. AY holds the Martin S. Kimmel Professorial Chair.

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