Review

Species-specific antibiotic-ribosome interactions: implications for drug development

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Abstract

In the cell, the protein synthetic machinery is a highly complex apparatus that offers many potential sites for functional interference and therefore represents a major target for antibiotics. The recent plethora of crystal structures of ribosomal subunits in complex with various antibiotics has provided unparalleled insight into their mode of interaction and inhibition. However, differences in the conformation, orientation and position of some of these drugs bound to ribosomal subunits of *Deinococcus radiodurans* (D50S) compared to Haloarcula marismortui (H50S) have raised questions regarding the species specificity of binding. Revisiting the structural data for the bacterial D50S-antibiotic complexes reveals that the mode of binding of the macrolides, ketolides, streptogramins and lincosamides is generally similar to that observed in the archaeal H50S structures. However, small discrepancies are observed, predominantly resulting from species-specific differences in the ribosomal proteins and rRNA constituting the drug-binding sites. Understanding how these small alterations at the binding site influence interaction with the drug will be essential for rational design of more potent inhibitors.

Keywords: antibiotics; drug; lincomycin; macrolides; protein synthesis; ribosome; streptogramin.

Introduction

The vital importance of translation within the cell makes the ribosome a major target for antibiotics (Gale et al., 1981). Crystal structures of the small or large ribosomal subunit in complex with antibiotics have revolutionised our understanding of their mechanism of action, as well as mode of interaction (reviewed by Wilson, 2004). The majority of these antibiotics have been shown to target the active centres on the ribosome, interacting predominantly with rRNA. The aminoglycosides, for example, interact with the decoding centre on the small subunit to induce misreading (Carter et al., 2000; Ogle et al., 2001), whereas the lincosamides, such as lincomycin, bind at the peptidyltransferase centre (PTC), the active site on the large ribosomal subunit, to inhibit substrate binding (Schluenzen et al., 2001; Tu et al., 2005b) (Figure 1A,B).

The macrolide antibiotics are a large and diverse class of antibiotics that have been shown to bind adjacent to the PTC within the tunnel of the large ribosomal subunit (Schluenzen et al., 2001; Schluenzen et al., 2003; Hansen et al., 2002; Tu et al., 2005b) and prevent the transit of the growing polypeptide chain as it passes through the tunnel during protein synthesis (Figure 1A; reviewed by Mankin, 2001; Jenni and Ban, 2003; Poehlsgaard and Douthwaite, 2003; Franceschi et al., 2004; Wilson, 2004).

Such insights are timely for the development of new and improved antibiotic derivatives in the never-ending fight against bacterial resistance, as well as in opening the way for rational drug design. Such an approach will require an intimate understanding of the relationship between the antibiotic and the target, the ribosome. One obvious limitation is the availability of very few target species upon which to base the study of these interactions. At present, the only suitable structures available include the small subunit from *Thermus thermophilus* at 3.0–3.3 Å (Schluenzen et al., 2000; Wimberly et al., 2000) and the large ribosomal subunit from the archaea *Haloarcula marismortui* at 2.4 Å (Ban et al., 2000) and from the bacteria *Deinococcus radiodurans* at 3.0 Å (Harms et al., 2001). Therefore, an important question is the extent to which the information yielded by the antibiotic-ribosome structures determined using these species is transferable to combat the pathogenic bacterial strains of interest.

Based on the observation that the regions targeted by antibiotics, such as the PTC, are phylogenetically highly conserved (Figure 1C), it might be reasonable to assume that antibiotics are likely to bind in a similar manner in all species. However, first comparisons between the binding modes of antibiotics on the large subunits of the archaea *H. marismortui* and bacteria *D. radiodurans* have suggested otherwise. Three 14-membered macrolide antibiotics, erythromycin, roxithromycin and clarithromycin, were first determined in complex with the *D. radiodurans* 50S subunit (D50S) (Schluenzen et al., 2001). Subsequently, the structures of 15- and 16-membered macrolide antibiotics, azithromycin, spiramycin, tylosin and carbomycin A, were solved on the *H. marismortui* 50S subunit (H50S), revealing that they have a similar location, orientation and conformation with respect to one another, but differ significantly from that observed for the 14-membered macrolides bound to D50S (Hansen et al., 2002). This was surprising considering the similarity between some of the macrolides; for example, azithromycin only differs from erythromycin by the absence of a keto-oxygen (C9) and the addition of a methyl-nitrogen at the C10 position (Figure 1D), yet the ribosome-bound conformations completely differ, with the lactone ring of...
Azithromycin adopting a folded-out conformation and erythromycin modelled into a folded-in conformation (Hansen et al., 2002).

One possible explanation for these differences is that wild-type archaeal ribosomes contain a guanine at position 2099 (2058 in E. coli (Ec) numbering] of the 23S rRNA, making them naturally resistant to macrolide antibiotics, whereas bacterial ribosomes, such as those of D. radiodurans, contain A2041 (EcA2058) and are thus susceptible to macrolides. Indeed, macrolide resistance in bacteria arises from A2058G (Ec) transitions that reduce the affinity of the drug for the ribosome (reviewed by Poehlsgaard and Douthwaite, 2003; Franceschi et al., 2004). To investigate this possibility, Steitz and co-workers produced H50S containing G2099A (Ec2058) mutations (Tu et al., 2005a). With the mutant ribosomes, it was thus possible to determine the structure of the H50S complexes with the 14-membered macrolides erythromycin and telithromycin, which were found to bind in similar positions and with similar conformations as the 15- and 16-membered macrolides (Tu et al., 2005b). In addition, azithromycin was found to bind to mutant and
Species-specific antibiotic-ribosome interactions

Table 1 Comparison of ribosome-antibiotic structures of the large subunit between archaea and bacteria.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Class</th>
<th>Species</th>
<th>Res. (Å)</th>
<th>PDB ID</th>
<th>Reference</th>
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<tr>
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<td>Azalide</td>
<td>D.r</td>
<td>3.2</td>
<td>1NWY</td>
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<td>1YHQ</td>
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<td>Lincosamide</td>
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</table>

* T. D.r and H.m correspond to the thermophilic bacterium *Thermus thermophilus*, the radiation-resistant bacterium *Deinococcus radiodurans* and the archaeabacterium *Haloarcula marismortui*, respectively. Wt indicates wild-type subunits were used as opposed to mutants as in footnote a.

b Res. value corresponds to the maximum resolution attained in the high-resolution bin.

c Protein data bank (PDB) files for each antibiotic complex can be downloaded at http://www.rcsb.org/pdb/.

d The structures for these antibiotics were solved in complex with *H. marismortui* ribosomes containing the mutation G2099A (position 2058 in *E. coli*).

e The complete structures for these D50S-antibiotics will be deposited with the D50S native structure (Schluenzen et al., manuscript in preparation); however, the coordinates for the antibiotic and surrounding ribosomal neighbourhood will be made available upon request.

wild-type H50S in a virtually identical way, thus supporting the invariability of macrolide binding to H50S, independent of whether position 2058 is adenine or guanine. However, the conformation was found to be distinctly dissimilar to the D50S-bound macrolides and further discrepancies were noted for the structures of clindamycin and the streptogramins between these two species. The antibiotic-ribosome structures for comparison between *H. marismortui* and *D. radiodurans* are compiled in Table 1.

There are a few possible sources for the apparent discrepancies between the antibiotic binding modes and conformations obtained for bacterial and archaeal ribosomes. These include: (i) species-specific differences, such as sequence and/or conformational variation in the rRNA nucleotides or r-proteins; (ii) environmental differences, resulting from the high-salt requirement of archaea, which could affect the conformation of the ligand and/or the ribosome; and (iii) partially incorrect or inaccurate interpretation of the electron density maps because of limited resolution and poor phasing models. To distinguish environmental or species-specific factors from artificial differences, we re-examined some of the previously reported antibiotic structures by: (i) taking advantage of a higher-resolution (currently at 2.89 Å) native D50S structure (Schluenzen et al., manuscript in preparation), which allowed the generation of more accurate difference maps; (ii) collecting additional data for antibiotic structures that had not been previously determined with D50S or to generate more accurate data sets; and (iii) applying a density modification procedure to reduce the background noise and enhance ligand density, the effectiveness of which was recently demonstrated by the modelling of *H. marismortui* L10 protein (Diaconu et al., 2005) from the electron density map of H50S (Ban et al., 2000).

Collectively, this approach allowed more precise comparison between the *D. radiodurans* and *H. marismortui* structures, which revealed that while the overall position and orientation of the antibiotics was in most cases remarkably similar, specific differences in the details of the interactions were observed. Here we present an overview of the main results, while specific details and revised structures will be presented together with the native structure (Schluenzen et al., manuscript in preparation). Perhaps the most striking difference is evident for the ketolide telithromycin, the side chain of which is clearly positioned in a completely different orientation in
both species. In addition, the shift in U2564 (EcU2585) induced by binding of the streptogramin dalfopristin to the \textit{D. radiodurans} large subunit does not occur in H50S, suggesting bacterial specificity. Furthermore, interactions for the lincosamides also differ, mainly because of a different orientation of A2062 (Ec) of the 23S rRNA. Finally, we note species-specific variation in the ribosomal proteins (r-proteins) L4, L22 and L32, which reach into the tunnel and scaffold rRNA within the region of the macrolide-binding site. Since the r-protein L32 has no homologe in \textit{H. marismortui} and differs in length and sequence for some bacteria, it may have an influence on macrolide binding to the tunnel and, together with other species-specific differences, provides an explanation for some of the differences between \textit{H. marismortui} and \textit{D. radiodurans} antibiotic-ribose structures.

\textbf{Similar binding modes for macrolide antibiotics}

To ascertain whether the macrolide antibiotics really bind in a species-specific manner to the large ribosomal subunit, we recently solved the structure of a derivative of the macrolide tylosin bound to D50S (Table 1), which allowed direct comparison with the previously determined structure for tylosin bound to H50S (Hansen et al., 2002). Tylosin is a macrolide antibiotic with a 16-membered lactone ring, a C5-mycaminose-mycarose disaccharide and a mycinose sugar at the C14 position (Figure 1D). The overall position and conformation of the lactone ring in the D50S-bound structure is almost identical to that of the macrolides found in the H50S structure (see I in Figure 2A). This is particularly remarkable, since the tylosin derivative used in this study contains a modification at the C6 ethyl aldehyde position (Sumbatyan et al., 2003), which consequently abolishes the ability to form a covalent bond with DrA2045 (Ec2062), as observed for A2103 (Ec2062) in H50S (Hansen et al., 2002). Instead, DrA2045 is rotated away without significantly altering macrolide binding. Similarly, the lactone ring is in the lower energy folded-out conformation, and the C5-disaccharide extends towards the PTC (Figure 2A I). One notable difference, however, is apparent for the position of the C13-C15 region of the lactone ring and C14-mycinose sugar, which are shifted relative to the H50S-tylosin structure (Figure 2A II). The shift most likely results from differences in domain II of the 23S rRNA, but possibly also L22, since the extension of this r-protein comes into close proximity to the mycinose sugar of tylosin. In the D50S structure, the side chain of Arg111 of L22 stacks along the mycinose sugar of tylosin (Figure 2A III). The equivalent position in \textit{H. marismortui} L17 (homologue of bacterial L22) is Met130, which stacks against the mycinose in a very similar manner (Figure 2A IV), and only requires a reorientation of the Met side chain compared to the native H50S, without affecting the conformation of the hairpin loop. Binding of tylosin to D50S requires not only a shift of Arg111, but also actually leads to a small conformational change of the tip of the \(\beta\)-hairpin loop (Figure 2A III).

Although the structural arrangement appears to be very similar in both species, the differences in domain II and protein DrL22/HmL17 clearly lead to different interactions with specific nucleotides of 23S rRNA. In H50S, the mycinose hydrogen bonds to the O2' of A844 (DrA764), whereas in D50S the altered orientation of the mycinose leads to hydrogen bonding of both O4C and O3C with the base of G761 (HmA841), thus closely mimicking a base-pairing scheme. Although H50S carries an adenine instead of a guanine at this position, it should still be competent to form interactions similar to those observed for D50S. However, the 23S rRNA of H50S contains an additional nucleotide inserted into H35 at position 835 (between Ec755 and 756), which leads to a shift of the nucleotides within this helix. This shift is propagated all the way up to A841 (DrG761) located in the loop of H35, with the end result that A841 is displaced by 1.2 Å towards the mycinose when compared to D50S (Figure 2A II). This displacement accounts for the differences in interactions observed between the mycinose sugar of tylosin and the rRNA in the D50S- and H50S-tylosin structures. Such a long-range alteration is reminiscent of resistant mutations, which frequently do not target the antibiotic binding site directly, but rather tackle remote structural elements (see below).

Having established that the binding mode of the 16-membered macrolide antibiotics is similar between \textit{D. radiodurans} and \textit{H. marismortui}, we were interested in addressing the 14-membered macrolides, such as telithromycin and erythromycin. Previous D50S structures for erythromycin and the erythromycin derivatives clarithromycin and roxithromycin were determined at rather limited resolution and were based on a structure of the native D50S lacking protein side chains (Schlünzen et al., 2001). The lactone rings of these structures were modelled in a higher energy folded-in conformation, differing significantly from the folded-out conformation observed for H50S-bound macrolides (Hansen et al., 2002, 2003; Tu et al., 2005b).

The revised structures for erythromycin and telithromycin on D50S are consistent with the folded-out conformation determined for H50S, as are the positions of the desosamine side chain and lactone ring. These D50S structures are also very similar in position and orientation to the structure of tylosin bound to D50S. This suggests that both 14- and 16-membered macrolide antibiotics bind similarly to archaeal and bacterial ribosomes, consistent with the high conservation within the 23S rRNA residues constituting the macrolide-binding site. However, careful analysis of the interactions of the antibiotics determined in complex with both H50S and D50S reveals a number of significant differences, which are outlined in the following sections for telithromycin, azithromycin, and the streptogramin and lincosamide classes of antibiotics.

\textbf{Variations in ketolide and azalide interaction with the ribosome}

The ketolide antibiotics are semi-synthetic derivatives of erythromycin, which display improved pharmacological properties and are even effective against some macrolide-resistant strains (Okamoto et al., 2001; Liu and...
Figure 2  Comparison of the binding positions of tylosin and telithromycin on archaeal and bacterial ribosomes. (A) Binding of tylosin to bacterial and archaeal ribosomes. I. Comparison of the overall binding position and conformation of tylosin (derivative) bound to the D50S (blue) and of tylosin bound to H50S (yellow; PDB1K9M; Hansen et al., 2002). II. The shifted position of nucleotides in H35 between D50S and H50S produces a corresponding shift in the position of the mycinose sugar of tylosin. *H. marismortui* rRNA nucleotides and H50S-bound tylosin are coloured grey and yellow, respectively, whereas *D. radiodurans* rRNA nucleotides and D50S-bound tylosin are coloured brown and blue, respectively. III. Binding of tylosin (derivative) to D50S induces a change in the conformation of the loop of L22 and especially of the side chain of Arg111 (N, native D50S L22, yellow, and T, tylosin-bound D50S L22, blue). IV. The mycinose sugar of tylosin stacks against the Met130 of L17 in the H50S (green) and the Arg111 of L22 in the D50S (cyan). (B) Comparison of the position and conformation of telithromycin bound to D50S (green) and H50S (yellow). Left: The electron density (meshed) for D50S-telithromycin revealing the different orientation of the heterocycle side chain of telithromycin (arrowed), as well as shifted positions of Dr2588 in domain IV and Dr765 in domain II of 23S rRNA. The equivalent positions of these nucleotides (C2644 and C845, respectively) in H50S (PDB1YIJ; Tu et al., 2005b) are shown in grey. Right: 90° rotation showing the interaction of the heterocycle side chain arm of telithromycin with nucleotides within domain II of 23S rRNA. A similar stacking interaction between the telithromycin side chain and Dr803 observed in D50S is not possible in the H50S structure because of the dramatically different position (arrowed) of equivalent nucleotide U883 (PDB1YIJ; Tu et al., 2005b).

Douthwaite, 2002; Franceschi et al., 2004). Like erythromycin, ketolides have a 14-membered lactone ring; however, unlike erythromycin, a cyclic carbamate is inserted at positions 11 and 12 and the cladinose sugar at the C3 position is replaced by a keto group, which explains the name derivation (Figure 1D). In addition, for the ketolide telithromycin, an alkyl-aryl side chain extends from the carbamate nitrogen.

The binding site of telithromycin on the ribosome reveals that this drug, like most macrolides, binds within the tunnel of the large ribosomal subunit (Berisio et al., 2003; Tu et al., 2005b). With regard to the revised structure of telithromycin (Table 1), there is now good agreement between the position of the lactone ring bound in the D50S and H50S structures – overlapping significantly with that of erythromycin – yet there still remains a striking discrepancy as to the orientation of the alkyl-aryl side chain (Figure 2B). Bound to the D50S, this heterocyclic side chain penetrates deeper into the tunnel, away from the PTC, where it interacts with nucleotides of domain II
of 23S rRNA (Berisio et al., 2003; Figures 1C and 2B). In contrast, in the H50S-telithromycin structure, the side chain is folded across the lactone ring and stacks on the base of C2644 (EcU2609) within domain IV of 23S rRNA (Tu et al., 2005b; Figure 2B).

The different positions of this moiety observed in the H50S- and D50S-telithromycin structures indicate that the side chain is highly mobile (Berisio et al., 2003; Tu et al., 2005b). Such flexibility allows the side chain to adopt the most energetically favourable position, which appears to be quite different between H50S and D50S. The stacking of the aromatic alkyl-aryl side chain of telithromycin evident in the H50S structure is prevented in D50S by the different orientation of U2588 (EcU2609) (see Figure 2B). Instead, in the D50S-telithromycin structure the aromatic side chain reaches down into the tunnel and inserts into a groove formed by nucleotides DrA764, A802 and C803 (EcA751, A789 and U790) within domain II of 23S rRNA, with the major contact comprising a stacking interaction of the telithromycin side chain against the base of C803 (Figure 2B). Since this region of 23S rRNA is poorly conserved and relatively flexible, it is not surprising that there are a number of differences in the sequence and structure between H50S and D50S. In particular, the equivalent nucleotide to C803 in H50S, U883, is rotated approximately 180° away from the C803 position and cannot make a stacking interaction as observed in D50S (Figure 2B). Together, the shifted positions of U2588 (HmC2644) and C803 (HmU883) explain why the most energetically favourable position for the telithromycin side chain is so different between H50S and D50S.

However, the biochemical protection and mutation data, predominantly determined in E. coli, associating telithromycin binding with domain II of 23S rRNA are not as easy to correlate with the structural data. Certainly, the predominant interaction of the alkyl-aryl arm of telithromycin is with C803 (EcA789; Figure 2B), yet in E. coli telithromycin protects A752 from chemical attack (Garza-Ramos et al., 2001) and deletion of this nucleotide confers ketolide resistance (500-fold increase in the minimal inhibitory concentration, MIC; Canu et al., 2002). We might therefore expect additional differences concerning some details of the binding mode in E. coli. In D50S, we note that upon telithromycin binding the base of C765Dr (A752Ec) becomes co-planar with U2588Dr (U2609Ec). Although the distance between the two bases is approximately 3.9 Å in D50S (Figure 2B), in E. coli U2609 and A752 are likely to form a base pair in the presence of ketolide antibiotics. This may suggest that the protection of A752 in E. coli results from base pair formation induced by telithromycin rather than direct interaction with A752. Consistently, the equivalent residue to A752 (DrC765) in D. radiodurans ribosomes is not protected by telithromycin binding (A. Mankin, personal communication). In E. coli, mutation of the neighbouring base U754A (DrG767) also confers resistance to telithromycin (Xiong et al., 1999), as does U2609C (Garza-Ramos et al., 2001; Figure 1C). DrG767 (Ec754) base pairs with DrC675 (Ec743) in H35 of 23S rRNA, and therefore it is easy to envisage that the U754A (Ec) mutation would disrupt H35 and thus indirectly affect residues located in the loop region that contact telithromycin (DrC759, U760 and A764). While deletion of A752 (Ec) would have a similar effect, the antibiotic resistance resulting from mutation of U2609C is harder to explain. In H50S, the U → C substitution should not affect the stacking interaction with the telithromycin side chain.

In summary, the differences in domain II between H50S and D50S can explain why in archaeal ribosomes, such as in H. marismortui, the side chain preferentially stacks against U2609 rather than penetrating deeper into the tunnel. However, we cannot exclude the possibility that the alkyl-aryl side chain of telithromycin adopts a different conformation in solution due to the high ionic buffer conditions, such that the differences in interaction with 23S rRNA are actually not prerequisite, but are rather induced by drug binding under different conditions. Furthermore, it raises the question as to whether the heterocyclic alkyl-aryl side chain of telithromycin would adopt yet other orientations in other species. In this respect, it would be interesting to investigate how the quinolylallyl group of the ketolide cethromycin (ABT-773), which also interacts with domain II of 23S rRNA in the D50S structure (Schlunzen et al., 2003), is oriented on H50S.

Other differences in antibiotic binding arising from differences in the domain II region of 23S rRNA are evident when comparing the available H50S- and D50S-azithromycin structures (Table 1). The azalide azithromycin is an erythromycin derivative with a 15-membered lactone ring, resulting from the insertion of methyl-substituted nitrogen at the C10 position. In contrast to the H50S-azithromycin structure (Hansen et al., 2002), two molecules of azithromycin were observed in the azithromycin-bound D50S structure (Schlunzen et al., 2003). One (Azi-1) is located in a position overlapping that of the previously determined macrolides and the other (Azi-2) is located directly adjacent to it, but deeper in the tunnel. In the second position, the azithromycin molecule is sandwiched between the loops of r-proteins L4 and L22, and makes contact with the primary Azi-1 position, as well as with RNA nucleotides located in domain II of 23S rRNA (Schlunzen et al., 2003). There may be coordinated binding of the two molecules, since the desosamine sugar of Azi-2 can hydrogen-bond directly with the lactone ring of Azi-1, and additional indirect contacts through a Mg ion are possible. However, the main contacts with Azi-2 occur between the loop of L4, specifically residues Gly60 and Thr64, which have the potential to form hydrogen bonds with the lactone ring and cladinose sugar of Azi-2, respectively. Since these positions are generally poorly conserved in bacteria, let alone other kingdoms, this may explain why a second binding position was not observed in the H50S azithromycin structure (Hansen et al., 2002). In addition, extensive hydrophobic interaction between Arg111 of L22, which is also specific for D. radiodurans, and the desosamine and cladinose sugars of Azi-2 was also observed. A kinetic analysis of the
binding of azithromycin to E. coli ribosomes has been performed and no evidence for a second binding site was reported (Dinos et al., 2001). This suggests that, at least for E. coli and H. marismortui, only one binding site on the 50S subunit exists for azithromycin. However, it does raise the question as to whether this is also the case for other organisms. If the binding of the azithromycin molecules were co-operative, this might open another avenue for the development of more potent inhibitors, i.e., those that work synergistically, as documented for the streptogramin antibiotics described next.

The synergistic and inhibitory mechanism of the streptogramin antibiotics

The streptogramin class of antibiotics consists of two structurally unrelated compounds, types A and B (Figure 1D), which act synergistically to inhibit translation (reviewed by Cocito et al., 1997). There is good agreement between the H50S- and D50S-bound streptogramin structures deposited (Hansen et al., 2003; Harms et al., 2004; Tu et al., 2005b; Table 1). In both structures, the streptogramin A (Sa) component binds at the peptidyltransferase centre (PTC), the active centre of the 50S subunit. The PTC is the site of peptide bond formation and contains two substrate-binding sites, the A site, where the aminoacylated tRNA binds, and the P site, where the peptidyl tRNA is located (Figure 1A). Sa overlaps predominantly with the A site, but also encroaches on the P site, consistent with the observation that Sa compounds have been reported to interfere with binding of both A and P site ligands (Chinalli et al., 1984). The streptogramin B (Sb) component binds directly adjacent to the PTC, located in the tunnel in a position overlapping the macrolide-binding site (Harms et al., 2004; Tu et al., 2005b) (Figure 1B). This is consistent with the observation that bacterial strains resistant to macrolide antibiotics also exhibit cross-resistance to lincosamycins and streptogramin B (so-called MLSb resistance; reviewed by Poehlsgaard and Douthwaite, 2003). Indeed, the macrolide erythromycin can effectively compete with Sa binding to the ribosome (Parfait et al., 1981) and MLSb resistance resulting from methylation or mutation at position A2058 (Pfister et al., 2004) can be explained by steric clashing with the C9-benzyl group of Sb (Tu et al., 2005b).

An important feature pertaining to the streptogramins is their unique ability to act synergistically. In isolation, the streptogramins exert a bacteriostatic effect on bacterial growth. However, in combination, lower drug concentrations are required and a bactericidal effect is induced (Cocito et al., 1997). Furthermore, in the presence of Sa, Sb compounds have been reported to bind to macrolide resistant ribosomes (E. coli A2058U) that do not bind Sa in the absence of Sb (Vannuffel et al., 1992). It was surprising, therefore, that poor density was observed for virginiamycin S (Sa) bound to the wild-type H50S [having G2099 (Ec2058)] in the presence of virginiamycin M (Sb) (Hansen et al., 2003). Admittedly, the high salt conditions (1.7 M) required for H50S crystallisation and soaking are far from the more physiological buffer conditions used to analyse the binding in E. coli (Vannuffel et al., 1992) and thus could be a contributing factor. Repeating the experiment with mutant H50S ribosomes (carrying the equivalent of EcA2058) allowed good density for both Sa and Sb compounds to be visualised (Tu et al., 2005b), similar to that observed previously with D50S (Harms et al., 2004). In each case, the reason for the synergistic interplay between the Sa and Sb antibiotics was immediately apparent. Firstly, there is a large interface for direct contact between the Sa and Sb components, comprising both hydrophobic and hydrogen bond interactions. Secondly, the streptogramins share contact with A2062 (Ec) of 23S rRNA, which is sandwiched between the conjugated amino group attached to the C18 of Sb and the macrocyclic ring of the Sa (Figure 3A I). Indeed, mutation of A2062 is among one of the very few rRNA modifications that gives rise to both Sa and Sb resistance (Depardieu and Courvalin, 2001). In both structures, A2062 moves from its position relative to that in the native structure (Figure 3A I). In the native H50S structure, A2103 (Ec2062) lies flat against the tunnel wall and binding of Sa alone sterically clashes with the base of A2103 (Ec2062), inducing it to rotate 90° into the tunnel lumen (Figure 3A I; Tu et al., 2005b). Movement in D50S and H50S of the equivalent base to EcA2062 is consistent with the observation that the binding of Sa to E. coli ribosomes protects A2062 from chemical attack and that subsequent binding of Sa makes it reactive again (Vannuffel et al., 1994).

A unique characteristic of the streptogramin A antibiotic is its prolonged inhibitory action following drug removal from the medium (Nysen et al., 1989; Parfait and Cocito, 1980). This persistence of growth inhibition in the absence of the antibiotic is presumed to result from stable perturbation of PTC (Canu and Leclercq, 2001; Vannuffel and Cocito, 1996). Indeed, the binding of Sa induces large-scale changes in many nucleotides within the PTC, such as U2585 and A2062 (Porse and Garrett, 1999; Rodriguez-Fonseca et al., 1995). However, it is still possible that the prolonged inhibitory activity results from a slow off-rate of the drug following antibiotic removal from the medium. In this case, the prolonged inhibitory activity is a direct consequence of the synergistic binding of the two streptogramin classes.

There is remarkable agreement in the binding positions of the Sa compounds between D50S and H50S (Figure 3A II). Despite this, only in D50S does binding of Sa alone, or in complex with Sb, lead to a dramatic change in the conformation of DrU2564 (EcU2585) (Figure 3A III–V). In the native D50S structure, U2564 is oriented towards the tunnel; on binding of the Sb dalfopristin, U2564 undergoes a 180° rotation to come into hydrogen-bond range of C2585 and G2567 (EcC2606 and EcG2588) (Figure 3A III; Harms et al., 2004). In contrast, binding of virginiamycin M to H50S does not induce any movement in C2620 (EcU2585). Although the ethanethiol moiety of dalfopristin, which is absent in virginiamycin M (Figure 1D), overlaps with the position of C2620 (Figure 3A V), this cannot be the reason for the lack of movement in H50S, since mikamycin (Table 1), which also lacks the ethanethiol moiety, also induces movement of U2585, albeit to a slightly lesser extent than dalfopristin (Figure 3A V). Furthermore, the hydrogen bond partners to the
Figure 3  Comparison of the binding positions of streptogramin and lincosamide antibiotics on archaeal and bacterial ribosomes.  
(A) I. The synergistic binding of the streptogramin antibiotics results from mutual interaction through A2062. The binding of quinupristin (S\textsubscript{a}, purple) induces a conformational change in Dr2045 (brown, EcA2062) compared with the native D50S structure (orange). The relative position of dalfopristin (S\textsubscript{b}) is shown in pink. In H50S, a similar position is evident for A2103 (yellow, Ec2062) upon binding of S\textsubscript{a} (quinupristin), alone (not shown) or in combination with S\textsubscript{b} (Virginiamycin M+S; PDB1YIT, Tu et al., 2005b). The position of A2103 in the native H50S is shown in green. II. Comparison of the relative binding positions of streptogramin A, dalfopristin (purple) and mikamycin (green) bound to D50S, with virginiamycin M (yellow) bound to H50S. III. The prolonged activity of the streptogramins results from movement of U2585. Left: binding of dalfopristin to D50S induces a dramatic movement (arrowed) of U2564 (EcU2585) from the native D50S position (orange) to the streptogramin-bound position (brown). Right: binding of virginiamycin M does not result in the movement of U2564 (EcU2585); compare the native H50S position (green) with the streptogramin-bound position (grey). IV. The position of the ethanethiol moiety of dalfopristin overlaps with the position of U2584 (EcU2585) in the native D50S (grey). V. The binding of mikamycin to D50S induces the movement of U2564 (EcU2585); compare the native H50S position (green) with the streptogramin-bound position (grey). The position of the ethanethiol moiety of dalfopristin overlaps with the position of U2584 (EcU2585) in the native D50S (grey). The relative position of virginiamycin M bound to H50S (PDB1YIT; Tu et al., 2005b) is shown for comparison. Electron density is shown as grey mesh in IV and V. (B) I. Clindamycin (blue) and lincomycin (pink) with electron density (for clindamycin, meshed) and relative position of H50S-bound clindamycin (yellow; Tu et al., 2005b). The difference in positioning of the propyl pyrrolidinyl moiety is arrowed. II. Relative positions of A2045 (EcA2062) for clindamycin (brown) and lincomycin (pink) D50S structures. The electron density (meshed) for clindamycin- and lincomycin-bound D50S structures is shown in blue and brown, respectively. The position of A2103 (EcA2062) in the clindamycin-bound H50S structure (Tu et al., 2005b) is shown for comparison. III. Possible coordination of an ion by clindamycin, ribose of U2564, and the bases of U2564 and U2485. Such coordination would not be possible for clindamycin bound to H50S (yellow) because of the alteration in the position of the equivalent rRNA nucleotides (grey).
rotated C2620 (EcU2585) in H50S are similarly oriented as in D50S, suggesting that if C2620 were to be flipped in H50S, this inactive conformation could, in principle, be stabilised. In this respect, it would be interesting to investigate the effect of dalfopristin binding to H50S on C2620 (EcU2585). Mutations of U2585 in E. coli are lethal in vivo and ribosomes carrying mutations at these positions show severely decreased (ca. 40-fold) release of the polypeptide in vitro (Youngman et al., 2004). This explains how a stable non-productive conformation of DrU2564 (EcU2585), such as that observed in the streptogramin-bound D50S structure, could induce the prolonged inhibitory activity characteristic of the S₆ class.

The difference in footprinting patterns on bacterial and archaeal ribosomes (Porse and Garrett, 1999) suggests that the mode of inhibition of streptogramin antibiotics may be different in these organisms. The absence of movement of C2620 (EcU2585) on S₆ binding to H50S may suggest that there is no prolonged post-antibiotic inhibitory effect in archaea, at least for some S₆ compounds; however, this remains to be tested. We note that occupation of the flipped DrU2564 (EcU2585) position in the dalfopristin-bound D50S was stronger than with mikamycin (compare Figure 3A IV and V), which might indicate that the post-antibiotic effect is stronger for S₆ antibiotics bearing side chains, such as ethanethiol, attached to the C26 position (Figure 1D). This site may therefore be important for further chemical modification. The quinuclidinylthio moiety has been shown to improve the inhibitory activity of quinupristin compared to virginiamycin S, which does not have this extension (see Figure 1D). Since the quinuclidinylthio moiety has weak density in the D50S complex, and makes minor contacts in both H50S and D50S structures (Harms et al., 2004; Tu et al., 2005b), the contribution of this moiety to the inhibitory activity is likely to be more important for solubility and uptake than for the binding affinity. Additional modifications at this position to induce higher affinity interaction with ribosomal components could further improve the effectiveness of this class of antibiotics.

**Lincosamides and the orientation of A2062**

The most prominent members of the lincosamide family of antibiotics are lincomycin and clindamycin, the latter being a semi-synthetic derivative of lincomycin with a hydroxyl group replaced by chlorine (Figure 1D). Despite the structural similarity, both compounds exhibit a slightly different protection pattern on 23S rRNA (Douthwaite, 1992) and clindamycin is the more effective drug in most cases (Spizek et al., 2004). The binding of clindamycin to the 50S subunit has been studied in both D. radiodurans and H. marismortui crystals, revealing a virtually identical placement of the drug within the PTC (Figure 3B I) (Schlüzen et al., 2001; Tu et al., 2005b), with the galactopyranosyl ring placed approximately in the same position as the desosamine of the macrolides (Figure 1B). In both structures, the pyrrolidinyl ring overlaps with the position of an A-site substrate, thereby efficiently inhibiting peptide bond formation. However, one difference proposed for the propyl pyrrolidinyl moiety of clindamycin in a rotation by ∼180° in H50S compared to the orientation observed in the D50S structure (as shown in Figure 3B I; Tu et al., 2005b). To verify this finding and to elucidate the structural differences between clindamycin and lincomycin binding, we investigated the structures of both lincosamides bound to D50S.

Neither of the available D50S or H50S structures (Table 1) shows any density for the propyl moiety and both conformations of the propyl pyrrolidinyl moiety of clindamycin fit the D50S electron density maps equally well (Figure 3B I). The conformation of the propyl pyrrolidinyl moiety as found in H50S is, however, in better agreement with the structure of the free molecule and leads to quite convincing interactions with the surrounding 23S rRNA (Tu et al., 2005b). The nitrogen linking the galactopyranosyl and pyrrolidinyl groups forms a hydrogen bond with the 2’OH of HmG2484 (EcG2505) and the carboxy oxygen is within 2.9 Å of HmA2103 (EcA2062), although the respective geometries disfavour hydrogen bonding. In addition, the O6 of HmG2102 (EcG2061) is within hydrogen bonding distance of the pyrrolidinyl nitrogen. From this type of pattern, we could expect clindamycin binding to strongly rigidify the local conformation of 23S rRNA, yet the temperature factors of the PTC indicate that nucleotides such as A2103 in H50S are destabilised on clindamycin binding.

The conformation of the D50S PTC in the presence of lincosamides differs from the conformation observed in H50S. DrG2484 (EcG2505), for example, is, slightly displaced compared to the equivalent H50S nucleotide (G2540), such that the proposed hydrogen bond to the peptidyl nitrogen of clindamycin (Tu et al., 2005b) is not possible, regardless of whether clindamycin assumes the cis or trans conformation. The most significant difference is observed for A2062 (Ec), which is known to attain dramatically different orientations, depending on the substrate bound (Hansen et al., 2002, 2003; Harms et al., 2004; Tu et al., 2005b), reminiscent of the flexibility observed for A2602 located at the PTC (see Wilson et al., 2005 and references therein). In the D50S-lincosamide structures, DrA2045 (EcA2062) is rotated by 90° compared to the orientation of A2103 (EcA2062) in H50S-clindamycin (and native H50S), and points directly towards the 3O of the galactopyranosyl ring (Figure 3B II). Although N6 of DrA2045 is too far away from the 3O for hydrogen bonding, the density is continuous between these two atoms (Figure 3B II). Since this region of the structure is very well resolved, the only plausible explanation is the presence of a water- or ion-mediated interaction between the N6 of DrA2045 and the 3O of the galactopyranosyl. The differences in the conformation of the PTC of D50S restrict specific interactions to the galactopyranosyl ring, whereas the pyrrolidinyl group provides additional stability by stacking between the base of G2044 (EcG2061) and the ribose of G2540 (EcG2505), independent of the cis or trans conformation of clindamycin. Therefore, it appears likely that both conformations of the propyl pyrrolidinyl tail of clindamycin shown in Figure 3B I are actually possible and both may even be present in ribosome-antibiotic structures. This
assumption also agrees with the complete lack of density for the propyl group and the variability of the propyl pyrrolidinyl tail between clindamycin and lincomycin, on and off the ribosome, as observed by NMR (Verdier et al., 2000). Curiously, this study actually proposed a conformation that represents an intermediate between those observed in the respective H50S and D50S crystal structures.

Investigation of a D50S-lincomycin complex revealed an almost identical picture in the density bridging DrA2045 (Ec2062) and the galactopyranosyl ring (Figure 3B I and II). There are very small differences in the precise positioning of lincomycin and clindamycin, and similarly small deviations within the PTC. Although some of these differences might simply be due to the coordinate error of working at 3.1–3.5 Å (Table 1), some differences definitely result from the presence of the chlorine in clindamycin, which is absent in lincomycin (Figure 1D). Together with the nucleotides U2485 and U2564, the chlorine of clindamycin clearly captures a divalent ion (Figure 3B III). The ion is completely absent in the lincomycin and native structures, and may therefore contribute to the stronger antimicrobial activity of clindamycin (Spizek et al., 2004). Curiously, the magnesium ion present in the D50S native and clindamycin structures, located between the phosphate-oxygens of C2420 (U2441Ec) and C2421 (C2442Ec) and the N3 of A2045 (EcA2062), is lost upon lincomycin binding, presumably due to the modest displacement (ca. 0.6 Å) of A2045. However, these peculiarities illustrate the structural differences resulting from the binding of near identical ligands rather than contributing to the specific inhibitory mechanism, and emphasise the difficulties of predicting how small structural changes to drugs will modify their mode of binding, a critical aspect of rational drug design.

Species-specific variation in ribosomal proteins influences antibiotic binding

The β-hairpin loop of ribosomal protein L22 encroaches the ribosomal tunnel and, together with the extensions of L4 and L32, defines the size of the tunnel kink close to where macrolide antibiotics bind (Figure 4A). The loop of L22 has been implicated in translational regulation (Tenson and Ehrenberg, 2002) and macrolide resistance (see below). As mentioned previously, in D. radiodurans r-protein L22, Arg111 comes into contact with the mycinose sugar of tylosin, which leads to a small conformational alteration of the L22 loop and simultaneously affects the positioning of the lactone ring (Figure 2A III and IV). Similarly, the side chain of Arg111 approaches the quinuclidinylthio moiety of quinupristin in the D50S complex (Harms et al., 2004) and causes a shift in its position relative to the H50S structure (Figure 4B; Tu et al., 2005b). Such differences between these organisms can lead to slight changes in the relative position of the antibiotic on the ribosome. Generally, pathogenic bacteria, and in fact most bacteria, have a positively charged residue at this position, usually Arg, Gln or Lys, whereas archaeal L17 generally have a hydrophobic residue, such as Phe, Trp and, to a lesser extent, Met, as observed in H. marismortui (Figure 4C). The lack of conservation in this area of L22, coupled with the slight differences in binding position of the mycinose sugar, suggests that rational design of antibiotics utilising the interactions within this region of the ribosome should be undertaken using ribosomes from the target organism, or as closely related an organism as possible.

Resistance to erythromycin in E. coli and Haemophilus influenzae strains arises from a deletion of three residues (Met-Lys-Arg, _MKR_ ) in L22 (Wittmann et al., 1973; Chittum and Champney, 1994; Clark et al., 2002). L22 is located lower in the tunnel than the macrolide binding site and L22 does not directly contact erythromycin (Schlünzen et al., 2001), consistent with the suggestion that the effect is indirect, operating through conformational changes in the rRNA (Gregory and Dahlberg, 1999). However, the deletion in L22 does not prevent the binding of erythromycin (Chittum and Champney, 1994), and therefore it is hard to envisage how resistance is conferred. To address this, Steitz and co-workers made the corresponding deletion mutant in L22 of H. marismortui and crystallised the mutant ribosomes (Tu et al., 2005b). The results suggest that the deletion detaches the loop of L22 from the tunnel wall, widening the constriction created by L4 and L22, directly below the macrolide binding site, in native ribosomes. The conclusion is that opening the construction somehow compensates for the occlusion created by the presence of erythromycin. However, we note that the erythromycin resistance phenotype in the bacteria _E. coli_ and _H. influenzae_ results from removal of the residues Met-Lys-Arg in L22 ( _MKR_ ) – a sequence that is relatively well conserved in bacteria – whereas in archaea the equivalent region is poorly conserved, with _H. marismortui_ having the sequence Gln-Gln-Gly ( _GSG_ ) (Figure 4C). Therefore, it is unclear whether such a deletion would even confer resistance to erythromycin [in tandem with a G2058A (Ec) substitution to overcome the natural erythromycin resistance in archaea].

One specific difference between bacterial and archaeal/eukaryotic ribosomes is the presence in bacteria of r-protein L32. In D50S, L32 has a small globular domain exposed to the solvent at the back of the subunit and a long 30-amino-acid N-terminal extension that penetrates through the ribosome to emerge in the tunnel. The extension converges at the tunnel kink and comes within 8–12 Å of the tip of the extensions of r-proteins L4 and L22, and encroaches on the macrolide-binding site (Figure 4A), such that the N-terminal Ala2 contacts the lactone ring of tylosin and the ribose of A2041 (EcA2058). Although the contribution to macrolide binding is not particularly important, L32 still influences the positioning of the lactone ring. While the desosamine is always positioned in an identical manner for all macrolides and ketolides, we observe a small but significant deviation of the lactone ring compared to the equivalent H50S structures.

More dramatic, however, is the unexpected conformational alteration of 23S rRNA resulting from the presence of L32. In H50S, which lacks L32, C2647 base pairs
Figure 4  Ribosomal proteins approach the macrolide binding site.
(A) The long extensions of L4 (red), L22 (cyan) and L32 (orange) converge at the tunnel kink, close to where macrolide antibiotics such as telithromycin (green) bind. (B) The side chain of Arg111 of L22 (yellow) approaches the quinuclidinylthio moiety of quinupristin (pink) in the D50S complex (Harms et al., 2004) and causes a shift in the position of this moiety relative to the position in the H50S structure (yellow; Tu et al., 2005b). The H50S position is possible because of the less extended conformation of Met130 at the equivalent position in L17 of H50S. (C) Sequence alignment of a region of archaeal ribosomal protein L17 with the bacterial homologue, L22. Highly conserved and similar residues are shaded black and grey, respectively. Deletion of MKR82 in E. coli L22 confers erythromycin resistance (equivalent regions in all species are boxed in orange). The green box highlights the positions equivalent to Arg111 in D. radiodurans L22 (DEIRA) and Met130 in H. marismortui L17 (HALMA). (D) His4 of L32 (yellow) overlaps with the relative position of U2647 (grey) in the H50S structure. The equivalent residue in D50S, C2591, is flipped out of the helix (arrowed) compared to the H50S position. His4 stacks on the base of DrU2590 (HmG2646), which base pairs with DrA2040 (HmC2098) in the D50S (and H50S) structures, and constitutes part of the macrolide (green) binding site. (E) Sequence alignment of the N-terminal region of bacterial-specific ribosomal protein L32. The variable region encompassing the first four amino acids is boxed (cyan). Highly conserved and similar residues are shaded black and grey, respectively. Note that the N-terminal Met1 that is post-translationally removed in some species is still referred to as the first residue.

with G2097 and stacks with G2646; however, in D50S, the same interactions are not possible, since His4 of L32 occupies this space (Figure 4D). His4 mimics an rRNA nucleotide by maintaining stacking interactions with the neighbouring nucleotide, in this case U2590. This may be important, since the base pair between U2590 and
A2040 forms a platform upon which the macrolide antibiotics sit. The presence of His4 sterically occludes the base and ribose of C2591, with the result that this nucleotide is flipped out of the helix with respect to the H50S position (Figure 4D). We envisage that in the absence of L32 (or the presence of an N-terminally truncated L32) C2591 would not be in a flipped position, but instead would be stacked within the helix and would base pair with G2039, analogous to the H50S C2647-G2097 base pair. In this respect it is interesting to note that the majority of bacterial L32 r-proteins are either truncated by three amino acids with respect to D. radiodurans L32, such as in E. coli, and therefore would be expected to have helical stacked base-pairing, as observed in the H50S structure. However, there are also a large number of bacteria in which the N-terminal Met1 residues substitute for the position equivalent to His4 in D50S, such as Mycobacterium tuberculosis (Figure 4E), for which it is hard to predict the effect on the surrounding rRNA.

To the future . . .

The continual emergence of multi-drug-resistant bacteria demands the discovery of new and more potent antibiotics. In the past 20 years, only one truly new antibiotic class targeting the ribosome has entered the market, revealing our dependence on the successive development of second- and third-generation derivatives. In the past, this has been successfully achieved and is exemplified by medical use of the semi-synthetic azalide and ketolide classes of antibiotics, which originated from macrolide parent compounds such as erythromycin. High-resolution information about the interaction of these antibiotics with the ribosome has brought the possibility of developing more potent derivatives using a rational approach. Comparative analysis of the binding of the macrolide, streptogramin and lincosamide classes of antibiotics to bacterial (D. radiodurans) and archaeal (H. marismortui) ribosomes revealed regions of the drugs that interact in a similar fashion with ribosomes of both species, as well as groups that exhibit remarkably different conformations and interaction patterns. Furthermore, a number of species-specific ribosomal components that exert an influence on drug binding account, at least partially, for the differential binding to bacterial and archaeal ribosomes. Therefore, the rational design of antibiotics should, wherever possible, utilise regions of high structural similarity or attempt to consider the implications of species-specific differences between the model organism used for drug development and the intended target organism.

Crystal structures of ribosome-functional complexes, such as initiation factor IF1 (Carter et al., 2001) and IF3 (Pioletti et al., 2003) on 30S and ribosome recycling factor (RRF) (Wilson et al., 2005) and trigger factor (Ferbitz et al., 2004; Baram et al., 2005; Schlünzen et al., 2005) on 50S, open another avenue for rational drug design, namely, the design of antibiotics that interfere with ligand binding, possibly by mimicking the interactions between the translation factor and the ribosome. In this context, the RRF may be an ideal target, since it is an essential translation factor in bacteria and is not present in the cytoplasm of eukaryotic organisms. The crystal structure of domain I of RRF bound to D50S suggests that the most important interactions are with H69 of 23S rRNA (Wilson et al., 2005), suggesting that disruption of these contacts may be lethal to the cell. Certainly, the de novo synthesis of antibiotic inhibitors that target unique regions of the ribosome will be one of the challenges for the future.

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References


