Crystal Structure of the Oxazolidinone Antibiotic Linezolid Bound to the 50S Ribosomal Subunit[||]

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Abstract: The oxazolidinone antibacterials target the 50S subunit of prokaryotic ribosomes. To gain insight into their mechanism of action, the crystal structure of the canonical oxazolidinone, linezolid, has been determined bound to the *Haloarcula marismortui* 50S subunit. Linezolid binds the 50S A-site, near the catalytic center, which suggests that inhibition involves competition with incoming A-site substrates. These results provide a structural basis for the discovery of improved oxazolidinones active against emerging drug-resistant clinical strains.

The oxazolidinone family represents one of only two new chemical classes of antibiotics disclosed in the past 40 years.[1,2] Linezolid (Figure 1), the first approved oxazolidinone, demonstrates good activity against all major pathogenic Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA[a]), vancomycin-resistant *Enterococcus faecium* (VREF), and penicillin-resistant *Streptococcus pneumoniae*.[3] However, not long after the commercial release of linezolid, oxazolidinone-resistant strains of MRSA and VREF began to appear in the clinic,[4,5] underscoring the increasingly urgent need for improved antibiotics that overcome bacterial resistance mechanisms. A particularly attractive goal would be a next-generation oxazolidinone with improved spectrum and binding affinity, while retaining properties for good oral exposure and safety.

The engineering of an improved oxazolidinone would be much easier if the mode of binding and mechanism of action were better understood. To this end, a variety of experimental methods have been used, often with surprisingly inconsistent results. Early biochemical studies suggested binding to the 30S ribosomal subunit or to areas of the 50S subunit not previously implicated in substrate binding.[6–8] More recently, the consensus is that oxazolidinones bind to the catalytic site on the 50S subunit. Most linezolid resistance mutations cluster around the ribosomal peptidyltransferase center (PTC), the site of peptide bond formation.[5,9–12] In addition, several photoreactive oxazolidinones have been cross-linked recently to the PTC in living bacteria, providing a low-resolution model of the binding site and binding orientation.[13] However, despite these advances in defining the linezolid binding site, it remains unclear exactly how the oxazolidinones work. A number of studies suggested inhibition of translation initiation, during which initiator tRNA charged with formylmethionine (fMet-tRNA^{fMet}) binds the 50S P-site, the binding site for peptidyl-tRNA.[7,8,14–16] Other studies, by contrast, have implicated inhibition of the elongation or termination steps of protein synthesis,[6,17,18] during which substrates may bind the P-site and the adjacent A-site, the binding site for incoming aminoacyl-tRNA. A definitive experiment to determine whether the oxazolidinones primarily inhibit P-site function, A-site function, or both functions is a worthwhile endeavor.

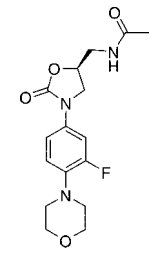

Figure 1. 2-D representation of linezolid.

As part of a structure-based program to discover and develop improved oxazolidinones, we have determined the crystal structure of linezolid bound to the 50S ribosomal subunit of *Haloarcula marismortui* (HMA) at a resolution of 2.7 Å. The structure reveals the atomic details of the interactions made between oxazolidinones and their ribosomal target site and yields insights into the structural basis for both the mechanism of translation inhibition by oxazolidinones and bacterial selectivity of this class.

Owing to uncertainty about linezolid's mechanism of action, it was not known a priori whether the drug would bind more tightly in the presence or absence of A- or P-site substrate analogues. Accordingly, structures of linezolid bound to H50S were determined with and without the addition of CCA-*N*-acetylphenylalanine (CCA-Phe), an analogue of the portion of aminoacyl and peptidyl tRNAs that interact most strongly with the 50S subunit. Notably, such CCA-Phe substrate analogues bind nearly equally well to the H50S A- and P-sites.[19] For the linezolid + CCA-Phe structure (PDB code 3CPW), difference experimental amplitudes were used to calculate the electron density map ($F_o - F_o$) to 2.7 Å. In the PTC, excellent density is seen for one linezolid molecule (Figure 2) and one CCA-Phe molecule, which is in the P-site. Additionally, very weak electron density corresponding to a second, partially occupied CCA-Phe molecule is observed in the A-site; it partly overlaps the stronger electron density for the linezolid molecule. Significantly, the binding conformation of linezolid in the presence of CCA-Phe does not differ from the conformation observed in the structure of linezolid alone (data not shown[20]). Moreover, the higher quality of the linezolid electron density determined in the presence of CCA-Phe suggests that the affinity of the drug may increase in the presence of the P-site substrate analogue. The H50S−linezolid−CCA-Phe complex has been refined to a final R_{free} and R of 0.2290 and 0.1904, respectively (data collection details and refinement statistics are given in the Supporting Information).

[||] PDB code for linezolid + CCA-Phe: 3CPW.

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[a] Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; VREF, vancomycin-resistant *Enterococcus faecium*; PTC, peptidyltransferase center; CCA-Phe, cytidine−cytidine−adenosine−*N*-acetylphenylalanine; H50S, G2099A mutant of *Haloarcula marismortui* 50S subunit.

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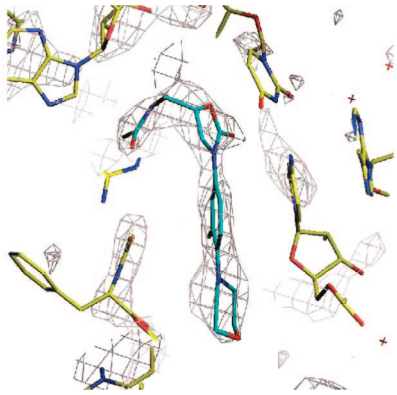

Figure 2. Unbiased experimental F_o(complex) − F_o(unliganded) map revealing electron density for linezolid (cyan) bound to the PTC of H50S.

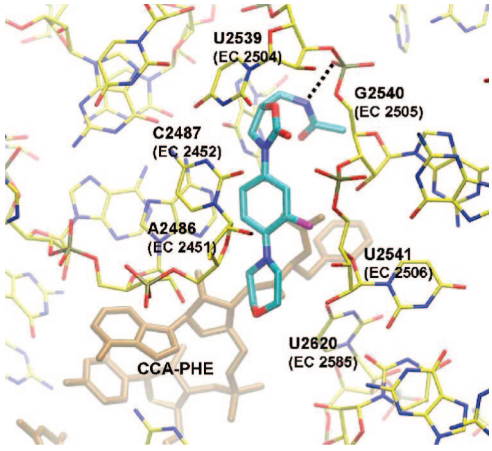

Figure 3. Overview of linezolid (cyan) and CCA-Phe (gold) binding to H50S. Residues with van der Waals interactions to linezolid are indicated, as is the acetamide arm hydrogen bond to G2540.

Linezolid uses hydrogen bonding and hydrophobic packing interactions to bind to an all-RNA pocket located within the ribosomal PTC. The oxazolidinone ring is stacked against the base moiety of U2539 (HMA numbering will be used throughout; Table S2 in Supporting Information gives the comparison with *E. coli*) (Figure 3), where it makes favorable van der Waals interactions. In addition, both the oxazolidinone ring and the C-5 acetamide arm show good shape complementarity with a portion of the ribosomal A-site surface located near the mouth of the exit tunnel. Further, the acetamide NH participates in a hydrogen bond with the phosphate group of G2540 (Figure 3). Published structure−activity relationships suggest an important role as hydrogen-bond acceptor for the ester-type oxygen in the oxazolidinone ring.[21] Yet, neither a directed hydrogen-bonding interaction nor a solvent- or ion-mediated one is apparent in the structure. Likely, the higher dipole moment of analogues featuring oxygen in this position drives the preference.

The fluorophenyl moiety of linezolid sits ideally in a heteroaromatic crevice formed by the PTC site residues A2486 and C2487, the so-called A-site cleft (Figure S1, Supporting Information). This is a wedge-shaped pocket in which the amino acid side chains of aminoacylated A-site tRNAs have been shown to bind and where several other antibiotics place hydrophobic functionality to inhibit function.[22] While the fluorophenyl ring of linezolid engages in a typical aromatic shifted-stacking interaction with C2487 (ring plane to ring plane distance is 3.5 Å; ring center to ring center distance is 4.3 Å), it participates simultaneously in an edge-to-face (or T-shaped)

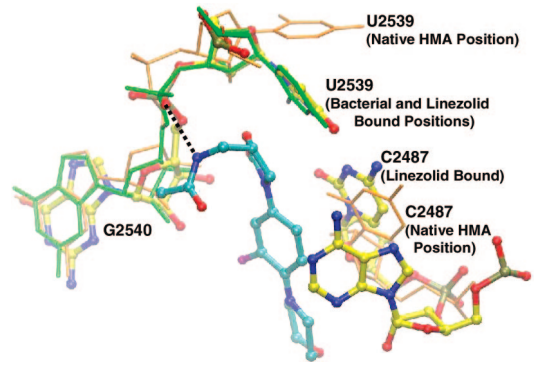

Figure 4. Residues U2539 and C2487 adopt different conformations from native positions (superimposed, orange) in response to linezolid binding.

interaction with the base of A2486. Both stacking and T-shaped interactions are common aromatic recognition elements in many host−guest systems.

The morpholino ring of linezolid does not appear to make significant interactions with the ribosome, which is consistent with the fact that many different functional groups can be substituted for the morpholine without a significant loss of activity.[21] From a drug-design perspective, this is a welcomed observation, for it points to a region of the molecule that can be "tuned" to adjust the pharmacological properties of the molecule without compromising the binding that is necessary for activity.

Comparison of the linezolid-bound and apo H50S structures reveals that ligand binding induces several significant conformational changes within the PTC. Linezolid binding causes residues U2539 and C2487 to rotate toward the lumen of the active site, allowing the formation of several important interactions with the ligand. For one, C2487 swings toward the drug, apparently to optimize the stacking interaction between the C2487 base and the fluorophenyl ring (Figure 4). This rotation also allows the fluorophenyl ring to complete a cascade of well-stacked aromatic rings including the nucleotides of C2487, A2488, and G2489 (Figure S1). This alteration of the A-site cleft has been observed as well when anisomycin ([(2*S*,3*R*,4*R*)-4-hydroxy-2-[(4-methoxyphenyl)methyl]pyrrolidin-3-yl]acetate) binds to the 50S subunit.[23] Linezolid binding also induces a large rotation of U2539 that results in stacking of its base with the oxazolidinone ring (Figure 4). This U2539 rotation alters the position of the G2540 phosphate group, allowing formation of the hydrogen bond with the acetamide arm of linezolid.

The induced-fit reorganization of the H50S active site provides useful insights into the structural basis for the prokaryotic selectivity of the oxazolidinone family. Comparison of the linezolid-bound structure of the archaeal H50S with the 2.8 Å resolution structure of the eubacterial 70S ribosome from *Thermus thermophilus*[24] reveals that the linezolid-bound conformation of H50S U2539 is nearly identical to the apo position of the homologous residue in the bacterial ribosome (Figure 4). This observation suggests that the ligand-bound conformation of the binding pocket for the oxazolidinone ring observed in H50S is preformed in bacterial ribosomes. Examination of other, lower-resolution bacterial 50S structures adds support for this hypothesis (data not shown; Rib-X unpublished).[25,26] In the bacterial structures, the uridine base appears to be constrained by a conserved adjacent adenosine base (EC A2572) to a "down" position, presumably with higher linezolid affinity. In the archaeal H50S structure, this bacterial adenosine is replaced by

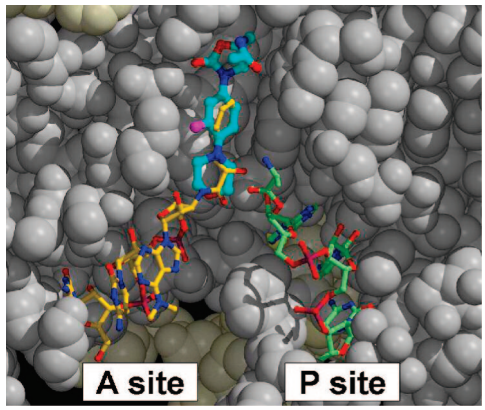

Figure 5. Superposition of the structure of linezolid (cyan) with the structures of A-site (orange) and P-site (green) substrate analogues bound to H50S determined previously (PDB entry 1VQN).[27]

a smaller uridine base (HMA U2607), which gives the archaeal residue the flexibility to occupy either native or linezolid-bound conformations. A smaller and possibly more rigid A-site pocket thus appears to be a feature of bacterial ribosomes, acting as a selectivity gate.

The flexibility of the H50S A-site pocket observed in these H50S crystal structures provides a model for the structural basis for the selectivity of anisomycin, an inhibitor of eukaryotic cytosolic ribosomes with no measurable activity against eubacterial translation. Anisomycin[22,23] binds to the same A-site pocket as the prokaryotic-specific inhibitor linezolid (Figure S2, Supporting Information). Superposition of the anisomycin and H50S−linezolid structures reveals that a portion of anisomycin would clash with the linezolid-bound and prokaryotic-like "down" conformation of U2539. This clash largely explains anisomycin's inability to inhibit bacterial ribosomes, and it also suggests that the larger A-site pocket observed in the apo H50S structure is a general feature of eukaryotic ribosomes exploited by a variety of other eukaryotic-specific inhibitors of the PTC. Beyond this, recent mutational and structural studies around the binding pocket of anisomycin suggest the overall plasticity of the region contributes to target potency.[23]

The linezolid-bound H50S crystal structure also provides a higher-resolution understanding of the structural basis for the mechanism of action of oxazolidinones. The 50S A-site has been identified in a crystal structure containing A-site and P-site substrate analogues bound simultaneously to H50S.[27] In this structure, the 50S A-site is defined by cytidine−cytidine−hydroxypuromycin (cytidine−cytidine−3′-[[(2*S*)-2-hydroxy-1-oxo-3-phenylpropyl]amino]-3′-deoxy-*N*,*N*-dimethyladenosine). In the H50S−linezolid structure, linezolid occupies virtually the same space as this A-site substrate ligand (Figure 5). Thus, it appears likely that oxazolidinones act by competing with substrates that bind to the ribosomal A-site. The fact that both linezolid and CCA-Phe bind the PTC simultaneously in the linezolid−H50S structure suggests that oxazolidinones do not interfere efficiently with binding of at least some peptidyl-tRNA substrates to the 50S P-site. This A-site binding mode makes it difficult to devise a simple model for how linezolid would block binding of fMet-tRNA^{fMet} to the P-site during initiation, as has been proposed.[8] Moreover, an A-site binding mode is also consistent with the results of genetic experiments, as well as lower-resolution RNA footprinting and cross-linking experiments of complexes of linezolid with bacterial 70S or 50S ribosomes.[6,28] Recently, Leach and colleagues[13] have constructed a model of linezolid binding based on results

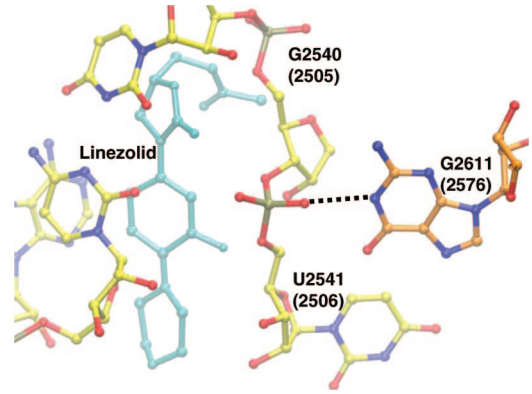

Figure 6. Mutation of G2611 to U, as found in ribosomes from linezolid-resistant clinical isolates, likely disrupts the stability of G2540 and U2541 to diminish affinity for linezolid (cyan).

obtained from in vivo cross-linking of linezolid derivatives to bacterial ribosomes. This low-resolution binding model appears to be in good overall agreement with the crystal structure of the 50S−linezolid complex reported here.

Crystal structures of antibacterial agents bound to their target sites often provide insight into the mechanisms that bacteria utilize to confer resistance to the agent. For example, a clinically important mutation common to MRSA and VREF linezolid-resistant strains is the substitution of the 50S nucleotide G2611 with uridine;[5,9−12] this resistance appeared shortly after the commercial launch of linezolid.[4,5] Of note, this mutation in HMA also confers resistance to anisomycin; a crystal structure of this mutant has been reported.[23] In the H50S−linezolid crystal structure, the uridine in question does not interact directly with linezolid. However, the base of G2611 interacts with the phosphate group of U2541, which along with the adjacent G2540 residue forms a major portion of the linezolid binding site (Figure 6). The substitution of G2611, a purine in the syn conformation, with a smaller uridine base that is less likely to adopt the syn conformation would remove the interaction with the U2541 backbone and destabilize the positions of G2540 and U2541. This destabilization thus would result in diminished linezolid affinity in the G2611U mutant ribosome, leading to the observed resistance.

The crystal structure of linezolid bound to the HMA 50S and an understanding of the high-value interactions available in the region encourage a structure-based design approach to next-generation oxazolidinones that overcome target-based resistance. To that end, we have designed and developed a series of enhanced oxazolidinones with overall greater target potency and antibacterial activity against pathogenic Gram-positive bacteria, including linezolid-resistant strains.[29] Unlike linezolid, these molecules also show good antibacterial activity against all the causative agents of community-acquired pneumoniae, including *Haemophilus influenzae* and *Moraxella catarrhalis*.

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Supporting Information Available: Methods of crystallization, data collection, and processing and structure refinement; additional references; Table S1 of data collection and refinement statistics;

Table S2 of key 23S rRNA residues in HMA and EC numbering; Figure S1 of cascade of π-stacking interactions; and Figure S2 of superposition of linezolid and anisomycin bound to the H50S A-site. This material is available free of charge via the Internet at http://pubs.acs.org.

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