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COMMUNICATIONS

Published on Web 02/28/2003

The Complex of a Designer Antibiotic with a Model Aminoacyl Site of the 30S Ribosomal Subunit Revealed by X-ray Crystallography

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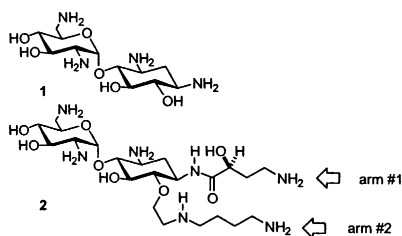
The bacterial ribosome is made up of over 50 proteins and three large RNA molecules that assemble to give the functional 70S ribosome, consisting of a 30S and a 50S subunit. The recent elucidation of the X-ray structures for these subunits and the intact 70S ribosome has created considerable excitement in the field that now adds structural insight to the mechanistic understanding that has been accumulating systematically over the past 40 years.[1]

The ribosome is an important target for several classes of antibiotics.[2] The "A site" on the 30S subunit is responsible for recognizing correct base pairing between the mRNA codon and the anticodon of the aminoacyl tRNA (decoding). This represents an important site for binding of many aminoglycoside antibiotics, which interfere with the decoding process and ultimately cause cell death. The clinical effectiveness of aminoglycosides has diminished due to bacterial resistance mechanisms such as enzymatic modification of aminoglycosides.[3]

We recently described a strategy in the structure-based design of novel antibiotics that bind to the bacterial A site.[4] For this exercise, we started with the structure of paromomycin bound to the A site RNA. The two rings of neamine (**1**) (a substructure of paromomycin), which itself lacks antibacterial activity (typical minimum inhibitory concentrations from 64 to <1000 μg/mL), were used as the template in our electronic search of other three-dimensional entities that would bind to the A site contiguously to the neamine structure. This resulted in a series of synthetic molecules that were shown to bind to a RNA A site construct by fluorescence measurements, some of which also possessed a potent broad spectrum of antibacterial activities. Compound **2** is one of these antibiotics.[4]



We report, herein, the X-ray structure of this antibiotic bound to A site RNA construct, first designed and solved by Vicens and Westhof,[5] and biochemical data showing that the translation machinery is the site of action. The 2.5 Å structure both validates the design paradigms for the antibiotic and reveals the molecular interactions made by this novel antibiotic in the bacterial A site.

Antibiotic **2** has excellent broad-spectrum activity against organisms such as *Escherichia coli*, *Serratia marcescens*, *Enterobacter cloacae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, including antibiotic-resistant variants. Inhibition of bacterial translation machinery by **2** was assessed in the MS2 translation assay, which measures the degree of incorporation of radioactive amino acids into polypeptide. This stringent assay uses *E. coli* extracts containing 70S ribosomes and MS2 phage RNA as a mRNA template. Antibiotic **2** shows a high degree of translation inhibition (>90% at 200 μM) in this assay. For comparison, amikacin, a clinically used antibiotic that inhibits ribosomal function by binding to the A site, shows intermediate activity in this assay (>70% at 200 μM).

Crystallization was performed as previously described[5] with some minor modifications.[6] The data were processed and scaled using the HKL2000 suite.[7] A molecular replacement solution was found using Molrep[8] with the structure of Vicens and Westhof[5] (PDB ID 1J7T) as the search model. This was refined in CNX,[9] using standard protocols. Where required, the model and ligand were manually built using O.[10] The crystallographic coordinates are deposited in the Brookhaven Protein Data Bank (1O9M).

The 2.5 Å crystal structure of the A site construct in complex with antibiotic **2** unambiguously defined the precise location for all atoms of antibiotic **2** within the A site (Figure 1). The neamine core binds in essentially the same manner as that observed in the A-site-paromomycin complex crystal structures, which was an assumption in the design. The unique arms of antibiotic **2** make different types of contacts with the RNA. This serves to validate the procedures and concepts used in its design.

The aminohydroxybutyryl group attached to the N1 position of neamine (arm #1) makes four contacts with the RNA. These interactions are dominated by involvement of the terminal amino group, which contacts the bases of G1403, G1498 (via water), and C1497. The hydroxyl group makes a water mediated contact to the sequence-independent phosphate backbone of U1495. The cavity in which arm #1 fits (at 12 o'clock in Figure 1B and C) is present identically in the structure of the intact 30S ribosomal subunit. Arm #2 (substitution at O6 position of neamine) also makes a number of interactions. The secondary amine makes a hydrogen bond with the N7 of G1405. The aliphatic portion of the arm has methylene hydrogens spaced between the phosphate backbone of residues C1404, G1405, and U1406. The terminal amino group also makes electrostatic interactions with the phosphate groups of G1405 and U1406 (Figure 1D).

This RNA construct contains two identical A-site motifs, each of which closely duplicates the structure of the A site in the ribosome. However, only one of the sites contains antibiotic **2**, while the empty site is involved in crystal packing interactions that prevent its occupation by the antibiotic. An unusual feature of the crystal packing in this crystal form is the displacement of U1406 from its U−U base pair to an extrahelical conformation, and in its place A1492 from the empty site comes in to form an A−U hoogsteen base pair (Figure 1A and B).

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10.1021/ja029736h CCC: $25.00 © 2003 American Chemical Society

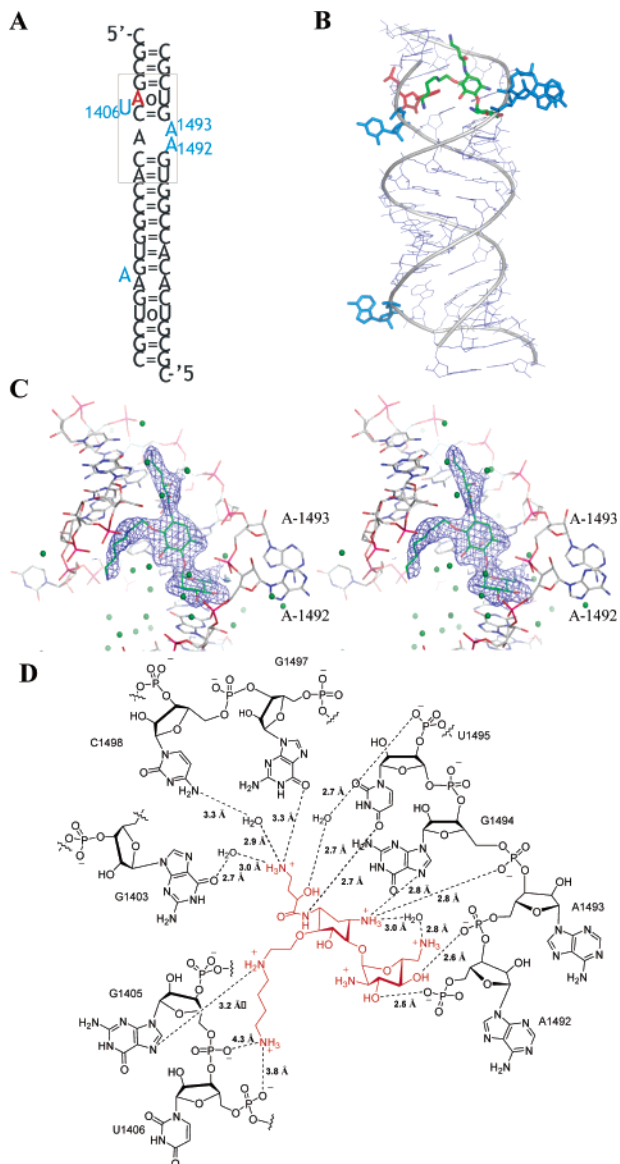

Figure 1. (A) Secondary structure of RNA in the crystal; box indicates bases forming the bacterial "A site". (B) Crystal structure of the complex. Extrahelical bases are shown in blue, and the symmetry related A1492 involved in A−U hoogsteen pair is shown in red. (C) Stereoview[13] of antibiotic **2** showing the experimental electron density at 1.5 σ ($2F_o - F_c$). The RNA carbons are shown in gray with the antibiotic **2** carbons shown in green; waters are shown as dark green spheres. (D) Schematic showing the interactions of antibiotic **2** (red) in the A site. The reported distances are for separations of heteroatoms.

A recent X-ray structure of the 30S subunit with an oligonucleotide for mRNA and the anticodon stem-loop of the cognate tRNA revealed that A1492 and A1493 had adopted a conformation out of the A site helix.[11] This extrahelical conformation allows specific interactions with the base pairs of the codon−anticodon and is proposed to be the molecular basis underlying decoding. The same extrahelical conformation of the bases was documented for binding of the aminoglycoside antibiotic paromomycin with both the 30S subunit[1] and the model for the A site[5] that has been used in our crystallographic efforts. The same conformation of A1492 and A1493 is also documented herein with antibiotic **2** bound to the A site, indicating that its molecular basis of action is the same as that of aminoglycosides with neamine as a structural component. The use of this model A-site construct is validated by the low rmsd (1.2 Å) for the ligand binding residues in this structure and those in common with the intact 30S subunit complexed with paromomycin.[11] A similar value was previously reported for the paromomycin complex of Vicens and Westhof.[5]

A significant drawback of aminoglycoside antibiotics is their susceptibility to bacterial resistance enzymes.[3] However, antibiotic **2** was previously shown not to be affected by a few important aminoglycoside-modifying enzymes.[4] This can now be rationalized in light of this crystal structure. Superimposition of antibiotic **2** onto the neamine core of kanamycin bound to aminoglycoside phosphotransferase APH(3′)-IIIa, one of the important resistance enzymes, reveals likely steric clashes between arm #1 and the aminoglycoside-binding loop of APH(3′)-IIIa.[12] This loop undergoes a conformational change upon substrate binding, and the presence of arm #1 would be expected to hinder the proper formation of the ternary complex. Additionally, the clinically used amikacin, which has a substitution similar to that of arm #1, is a poor substrate for this modifying enzyme, suggesting a route for modification of the core aminoglycoside structure to overcome one aspect of bacterial resistance.

To conclude, the crystal structure of antibiotic **2** in complex with the bacterial A site presented herein provides a detailed view of the molecular interactions underlying its mechanism of action combined with the characteristics necessary for the avoidance of modification by resistance enzymes.

Acknowledgment. We thank Julieanne Tester, Gulin Guler-Gane, and staff at the European Synchrotron Radiation Facility (ID29, ESRF), Grenoble, for technical assistance.

Supporting Information Available: Data processing and refinement statistics (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(6) The RNA (CGCGUCACACCGGUGAAGUCGC) [Dharmacon Research] (2 mM) was exchanged into 25 mM NaCl, 50 mM NaCac, pH 6.4, 2.6 mM MgSO4, 63 μM EDTA and annealed by heating to 90 °C and slowly cooled to room temperature to form the crystallization duplex (1 mM). The complex was formed by the addition of antibiotic **2** (2 mM) dissolved in 200 mM NaCac, pH 6.4, 300 mM KCl to the annealed RNA to form a 1:1 mixture. Crystallization was performed at 37 °C using hanging drops over a reservoir of 40% MPD. The drops were formed by combining 1 μL of complex and 1 μL of crystallization solution (1−5% of both MPD and Glycerol [Fluka]). Clusters of thick hexagonal plates (150 × 150 × 40 μm) grew in 1.8% MPD and 1.8% glycerol. The crystals were bathed in cryoprotectant (40% MPD, 12.5 mM NaCl, 125 mM NaCac, pH 6.4, 1.28 mM MgSO4, 0.031 mM EDTA, and 150 mM KCl) for 1−10 min prior to flash cooling with liquid nitrogen.

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JA029736H