The Search for New Antibiotics
Targeting the 50S Ribozyme

Structural details for 50S ribosome plus an analytic model help investigators to make, evaluate compounds that block protein synthesis

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To ensure that genetic information is reliably transmitted to daughter cells and converted to protein, cells contain sophisticated molecular machines for replicating DNA, transcribing it into messenger RNA (mRNA), and translating the information encoded in mRNA to make proteins. Each step takes place at a different rate, with DNA polymerase producing new DNA strands at about 1,000 nucleotides per second, whereas RNA polymerases yield 50–100 nucleotides per second. Ribosomes catalyze mRNA-directed protein synthesis in all living cells, linking about 15 amino acids per second.

The structure of the ribosome not only reflects the functional complexity of protein synthesis, but is unique in that RNA molecules rather than proteins catalyze this process. All ribosomes consist of two subunits, which are denoted 30S and 50S in bacteria or 40S and 60S in eukaryotes.

The smaller 30S subunit consists of one 16S rRNA molecule and about 20 proteins, and plays an important role in decoding mRNA while maintaining translational accuracy. The larger 50S subunit consists of two rRNA molecules (5S and 23S) and about 32 proteins, and catalyzes peptide bond formation. Although the 30S binds its substrate mRNA independent of its association with the 50S subunit, the 70S ribosome (or 80S ribosome in eukaryotes) simultaneously processes the tRNA substrates that supply amino acids for the growing polypeptide chains. Consistent with the macromolecular dimensions of its substrates, the ribosome is very large, dwarfing other molecular machines, including those that replicate DNA and produce molecules of mRNA.

History of Ribosome Crystals

Early attempts to analyze ribosomal structures focused on ribosomal crystalline arrays. These efforts began in the 1960s, when researchers used electron microscopy to observe flexible sheets of 80S ribosomes in a two-dimensional lattice of the \(p4\) plane group, after cooling chick embryos to 10–15°C. Similar ribosome crystals were observed in the neurons of chick embryos and the ovarian cells of lizards. In all cases, the crystalline arrays were formed from tetramers of 80S ribosomes. Single sheets of tetramers form in chick embryos, whereas the sheets of ribosomes that develop during winter hibernation in oocytes of the lizard \(Lacerta sicula\) are double-layered, forming from tetramers that attach to membranes, with the large subunit being juxtaposed to the membrane.

This early observation of large subunits being attached to membranes has been more clearly revealed in recent electron cryomicroscopy of eukaryotic ribosome-channel complexes engaged in de novo protein secretion. After better means for reconstructing three-dimensional images became available, researchers examining ribosomes from chick embryos detected a tunnel in the large ribosomal subunit that appears to originate from the site of peptide bond formation and extends through the opposite side of the particle. The diameter and length of the tunnel appear large enough to accommodate a peptide containing about 40 amino acids, consistent with the estimated size of the peptide.
protected by the ribosome, as determined by biochemical experiments.

Attempts to solve the structures of ribosomal microcrystals from 50S subunits of *Escherichia coli*, *Bacillus stearothermophilus*, *Thermus thermophilus*, and *Haloarcula marismortui* followed. Investigators found that their efforts to form crystals depended on the bacterial strain from which the ribosomes were isolated, the functional state of those ribosomes, and fine-tuning of crystallization conditions. However, even with good crystals, investigators could not hope to produce high-resolution structures until there were advances in X-ray crystallography such as brighter and more-focused synchrotron X-ray sources, larger and faster X-ray detectors, as well as advances in crystallographic software and computers. These advances, combined with the ability to generate suitably pure ribosomal subunits that, in turn, yielded crystals stable to brighter beams, allowed the application of data collection and phasing strategies for the large-unit cells that 50S subunits form.

By 2000, Thomas Steitz, Peter Moore, and their collaborators at Yale University in New Haven, Conn., determined the *H. marismortui* 50S subunit structure to a resolution of 2.4 Å. Determining this structure and subsequent analyses laid to rest doubts about whether the ribosome functions as a ribozyme, an idea championed years ago by Harry Noller at the University of California, Santa Cruz.

**The Ribosome as a Drug Target**

The availability of this structure and analyses of subsequent antibiotic bound-50S structures prompted a group of scientists and investors in 2001 to found Rib-X Pharmaceuticals (for ribosome X-ray crystallography). The ribosome is a particularly well-validated target for drugs with which to combat bacterial pathogens. Several different classes of chemical compounds specif-
ically inhibit bacterial protein synthesis, including clinically proven antibiotics such as azithromycin (Zithromax®) and clarithromycin (Biaxin®). There is a large body of structure-activity relationships (SAR) for many of these antibiotics plus a great deal of genetic and biochemical data describing structure-function relationships of the ribosome.

The ribosome structure is highly conserved among pathogenic bacteria and, although conserved throughout kingdoms, enough differences between ribosomes of bacterial pathogens and their human hosts exist to allow the design of selective agents. Most of the early ribosomal inhibitors work by interfering with the RNA component of the ribosome. However, without a high-resolution structure of the ribosome, it was not possible to address important questions about either the fundamental mechanisms of translation or the mechanism of action of ribosome inhibitors. Thus, until recently, the development of new antibacterials targeting the ribosome had been pursued without the crucial insights that a high-resolution structure of the target provides.

In addition to the coordinates of the native 50S structure from Haloarcula marismortui and complexes it forms with some antibiotics, we have developed proprietary X-ray crystallographic knowledge from studies of additional antibiotics that are proprietary to Rib-X. To keep our approach efficient, we have industrialized our approach to preparing ribosomal crystals, while we also optimized other protocols for reproducibly obtaining structures of antibiotic-ribosomal complexes.

Our drug discovery approach is one of structure-based drug design (SBDD) using proprietary tools and structures to fuel the engine. Structure-based design rests on the notion that one can “see” how a molecule or inhibitor binds to the target, thus facilitating computational analysis of other factors that contribute to binding, such as shape and electrostatic complementarity. Together, these factors can be used to streamline medicinal chemistry efforts to optimize compounds that bind more tightly to the ribosome and that circumvent known mutations that lead to resistance to established antibiotics.

The concept of SBDD is at least 20 years old, but some of the fruits of SBDD have only recently been realized. Drugs on the market such as nelfinavir (Viracept®), celecoxib (Celebrex®), and dorzolamide hydrochloride (Trusopt®) can trace their origins to structure-based analytic methods. Recent major advances in crystallographic techniques and in software and hardware speed and sophistication, coupled with the realization that successful drug design involves much more than making more potent binders, has made structure-based design an even more attractive tool for discovering drugs.

To help us interpret how novel compounds interact with and possibly inhibit the 50S crystal structures, we use a suite of proprietary computational tools (Analog™). Analog™ is a novel de novo design software package initially developed by another of our co-founders, William L. Jorgensen of Yale. It simulates constructing individual novel molecules or derivatives when it is provided with data for a particular core molecule such as a macrolide, and then is programmed to add substituents.
Rather than docking blindly a library of compounds into a presumed active site or developing a statistical model based on two-dimensional molecular factors describing activity, Analog™ uses structural information based on crystallographic analysis of lead compounds that bind the 50S subunit. For example, the program can seek a replacement for the macrocyclic ring of a macrolide by extracting physicochemical features of “reporter” compounds important for affinity and then suggesting replacements that are more synthetically tractable (Fig. 1).

In addition to rank-ordering analogs by how well they bind and fill targeted space in the ribosome, compounds are enriched for drug-like properties such as oral bioavailability, good solubility, and minimized activity in the central nervous system (CNS). This process involves an algorithm called the fully integrated ADME (absorption, distribution, metabolism, excretion) calculator, or QikProp. Calculations are made on unbound and bound molecules, both of which are important to assess because candidate drugs take different forms when in host or bacterial environments.

A major key to using SBDD successfully lies in the iterative solutions of high-resolution structures to which antibiotics are bound. Although analyzing a single compound that binds to the 50S can provide insight, analyzing many compounds, including related structures, provides an ever-growing knowledge of small molecule-RNA themes. This knowledge set, along with biological data from compounds, is then used to design new compounds and set priorities for evaluating them further. As the database is enriched, models can be built to predict compounds that will embody particular attributes. A long-sought model is one that would enrich for compounds with activity against gram-negative pathogens such as *Hae-mophilus influenzae*. We recently devised a consensus model for this pathogen that we now use routinely to rank-order compounds.

**Atomic Structures Unravel Mechanisms, Providing Clues to Circumvent Resistance**

Antimicrobial resistance continues to increase in hospital and community settings, reducing options for treating patients. When resistance leads to therapeutic failures, physicians are forced to use increasingly costly and toxic antimicrobials and hospital stays often are extended, increasing health care costs while facing patients with increased risk of morbidity and mortality. Moreover, because patents for antibiotics such as axoxicillin/clavulanate (Augmentin®), ciprofloxacin (Cipro®), and azithromycin (Zithro-
max®) have or soon will expire (in December 2002, December 2003, and November 2005, respectively), the marketplace likely will see a rise in the number of generic products that could lead to increased and perhaps inappropriate uses of these drugs, risking a sharper rise in resistance despite educational efforts to forestall those risks.

We need to understand the impact of target-based resistance and what our options are for designing antibiotics that circumvent resistance. Resistance to 50S inhibitors containing macrolide structures (Fig. 2) such as erythromycin (14-membered), azithromycin (15-membered), and tylosin (16-membered) developed following their continued clinical use.

Each of these antibiotics contains a macrocyclic ring to which an amino sugar attaches at the C5 position. The hydroxyl groups decorating the macrocyclic lactone orient themselves on one face of the ring structure, while the more hydrophobic face contains the hydrogens. The hydrophobic face is found nestled against the peptide tunnel wall in the 50S, where it has van der Waals and shape interactions to stabilize it. The sugar(s) at C5 extend “up the tunnel” toward the peptidyl transferase site of 50S (Fig. 3).

The atomic resolution of these structures explains a great deal of biochemical data, some of it 35 years old. For instance, when ribosomes are inhibited by 14- and 15-membered macrolides, they continue to synthesize longer peptides than when they are exposed to 16-membered macrolides—largely because most naturally occurring 16-membered macrolides contain two sugars at the C5 position, thereby better blocking protein synthesis at an earlier stage. Thus, peptides containing only 2–4 amino acids are made in the presence of 16-membered macrolides, while peptides of up to 8 amino acids can be made in the presence of erythromycin.

The three-dimensional structures showing 16-membered macrolides complexed to the 50S subunit of H. marismortui 50S also reveal several things about macrolide resistance that biochemical experiments missed. One such example depends on a previously unrecognized covalent attachment of the ethyl aldehyde at the C6 position to the adenosine at position 2062 in 23S rRNA (Escherichia coli numbering). This carbinolamine bond explains how a clinical isolate of Streptococcus pneumoniae could be resistant to 16-membered, but not 14- or 15-membered, macrolides. Thus, in this particular isolate, the adenine at 2062 had mutated to a cytosine, making bond formation unlikely.

Another example involves Erm methylases, which mediate a widespread mechanism of resistance to macrolides. These enzymes transfer one or two methyl groups to A2058, conferring resistance to all types of macrolides. Using our computational programs to analyze how methylation of this residue confers macrolide resistance, we could see that methylation would break a crucial hydrogen bond common to all the macrolides between the N1 position of adenine 2058 and the 2’-hydroxy of the amino sugar. Mutations that change adenine to guanine at this position result in macrolide resistance because the exogenous amino group of guanine thoroughly disrupts the interaction of

**FIGURE 4**

Space-filling model of novel gram-positive 50S subunit. (A) View from the 30S. (B) Back of the 50S subunit. tRNA residues (gray); ribosomal proteins (blue). Structure-based drug design at Rib-X is a ligand-driven process that starts with the crystallographic solution of a compound complexed to H. marismortui 50S subunit (step 1). As an example, we use Analog™, our proprietary computational software, to look for a new scaffold to replace the macrocyclic ring of the macrolide, carbomycin A (step 2). Possible analogs are suggested based on physicochemical properties and binding affinities of reporter molecules (multiple macrolides bind in this space) and the analogs are rank-ordered based on general fit and affinity. The analogs are tested both in the context of the ribosome and in the unbound state (steps 3–5). QikProp and other predictive filters are used in tandem to enrich for compounds with desirable drug-like properties (step 6). Compounds are tested in biological assays and data is used to build smarter predictive models.
the hydrophobic face of the antibiotic with the tunnel wall. The macrolide reacts as anyone would when poked with an elbow in the back. These and other subtle changes are captured in the complexes we solve and provide us with important clues as to how to design new molecules that circumvent these modifications.

### Early Compounds Demonstrate the Success of Our Approach

As a scientist in antibacterial drug discovery for more than 20 years, I find it extremely gratifying to see how well the ligand-driven SBDD approach works. For instance, in one program at Rib-X we tested the in vitro whole-cell activity of a series of compounds against a panel of respiratory tract (RT) pathogens (see table). The panel included clinical strains of *Streptococcus pneumoniae* with different characterized resistance mutations to macrolides and ketolides, penicillin, chloramphenicol, and trimethoprim-sulphamethoxazole. Several compounds, A-C, which were among the earliest compounds made in the series, have remarkable MIC activities.

In a second round of synthesis, we produced compound D, and it showed a four- to eight-fold increase in activity versus *Haemophilus influenzae*, a gram-negative pathogen. Compounds E and F from a third round of synthesis provided a balance of activities against the multidrug-resistant pneumococci and *H. influenzae*, both of which are key respiratory tract pathogens.

The ultimate test to determine if our approach is providing compounds with drug-like properties is whether any of them protect animals against pathogens challenges. We tested compounds E and F in a murine peritonitis model with macrolide-resistant *S. pneumoniae* 02J1175 as a challenge organism. Both (oral PD$_{50}$s of 23.3 and 12.0 mg/kg; IV PD$_{50}$s of 0.47 and 0.60 mg/kg for compounds E and F, respectively) were more effective than Zithromax® (oral PD$_{50}$ = 200 mg/kg and IV PD$_{50}$ = 14.2 mg/kg) or telethromycin (Ketek®) (oral PD$_{50}$ = 43.5 mg/kg and IV PD$_{50}$ = 2.6 mg/kg) when administered either orally or intravenously. Thus, leveraging structural information on how compounds bind to the 50S subunit is allowing us to identify potent, efficacious compounds.

### The Future of Drug Design

*H. marismortui* is a halophilic extremophilic bacterium belonging to the kingdom *Archaea*. Crystal structures of purified 50S ribosomal subunits from this species are providing us with useful guidance and insights into structures for drug design. Moreover, our tests of several novel compounds are validating this approach, and several of these molecules proved potent in vitro and in vivo.

Because *H. marismortui* 50S has some characteristics of eukaryotic 60S as well as bacterial 50S, this has allowed us to solve structures with compounds selective for either eukaryotic or prokaryotic ribosomes or both. By interpreting how compounds selective for eukaryotic ribosomes bind, we can “reverse engineer” leads and test for binding to prokaryotic ribosomes. In addition, colleagues at Rib-X recently solved the high-resolution structure of the 50S subunit from a gram-positive species (Fig. 4) that will enable us to understand some important nuances about bacterial pathogens that neither *H. marismortui* nor *Deinococcus radiodurans* 50S structures can serve to elucidate.

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The Microbial Challenge: Human-Microbe Interactions

Author: Robert I. Krasner, Providence College

This accessible and fascinating book on human-microbe interactions is the perfect text for use in undergraduate science courses. Designed to help students better understand the biology of the microbial world and its effect on their lives, this timely volume covers issues of vital importance, including biological warfare and terrorism, antibiotic resistance, the global impact of microbial diseases, and immunization.

A hybrid of microbiology and public health, The Microbial Challenge emphasizes the significance of microbes in everyday living. Students are led to understand public health problems and are provided a greater awareness of disease on a global scale through an examination of microbial (infectious) diseases and their societal consequences, including descriptions of some of the major microbial diseases through the ages, efforts put forth to meet the challenges raised by microbes, and public health measures of protection and surveillance put in place to keep ever-challenging microbes at bay. The beneficial nature of microbes is also examined; they are vital to the cycles of nature, play an important role in the food industry, and are significant tools in biological research.

Richly illustrated with many photos from the author’s extensive personal collection taken during his numerous trips overseas, The Microbial Challenge is ideal for students not majoring in science, for allied health sciences courses, and for public health courses. It can also be used as supplementary reading in standard microbiology and other biology courses.


List and ASM member price: $89.95