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Using Sequence-Specific Oligonucleotides To Inhibit Bacterial rRNA

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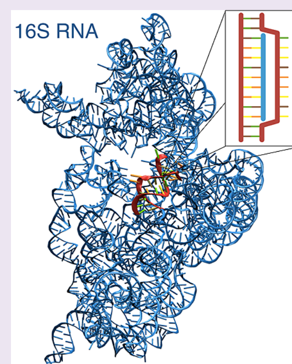
ABSTRACT: The majority of antibiotics used in the clinic target bacterial protein synthesis. However, the widespread emergence of bacterial resistance to existing drugs creates a need to discover or develop new therapeutic agents. Ribosomal RNA (rRNA) has been a target for numerous antibiotics that bind to functional rRNA regions such as the peptidyl transferase center, polypeptide exit tunnel, and tRNA binding sites. Even though the atomic resolution structures of many ribosome−antibiotic complexes have been solved, improving the ribosome-acting drugs is difficult because the large rRNA has a complicated 3D architecture and is surrounded by numerous proteins. Computational approaches, such as structure-based design, often fail when applied to rRNA binders because electrostatics dominate the interactions and the effect of ions and bridging waters is difficult to account for in the scoring functions. Improving the classical anti-ribosomal agents has not proven particularly successful and has not kept pace with acquired resistance. So one needs to look for other ways to combat the ribosomes, finding either new rRNA targets or totally different compounds. There have been some efforts to design translation inhibitors that act on the basis of the sequence-specific hybridization properties of nucleic acid bases. Indeed oligonucleotides hybridizing with functional regions of rRNA have been shown to inhibit translation. Also, some peptides have been shown to be reasonable inhibitors. In this review we describe these nonconventional approaches to screening for ribosome inhibition and function of particular rRNA regions. We discuss inhibitors against rRNA that may be designed according to nucleotide sequence and higher order structure.



Many known antibiotics, both natural and synthetic, interfere with protein synthesis in pathogenic bacteria by inhibiting ribosome function.[1] The bacterial ribosome is a 2.5 MDa protein−RNA two-subunit complex, responsible for the production of proteins in the cell.[2] Ribosomal RNA (rRNA) constitutes approximately two-thirds of the ribosome's molecular weight. Not surprisingly, rRNA not only provides the structural skeleton but also contributes to the ribosome function, and a vast majority of drugs work by interacting directly with rRNA and inhibiting its function.[1,3]

The subunits in the bacterial ribosome are named 30S (small subunit) and 50S (large subunit) in accordance with their sedimentation coefficients. The rRNA of the 30S subunit is named 16S RNA (over 1500 nucleotides long), and that of the 50S subunit 23S RNA and 5S RNA (about 2900 and 120 nucleotide-long chains, respectively). The ribosome translates the nucleotide sequence of messenger RNA (mRNA) to synthesize the protein, by incorporating the amino acid carried by the appropriate transfer RNA (tRNA). During translation each tRNA passes through three ribosomal binding sites, each positioned in both subunits, designated A, P, and E. The 30S subunit is responsible for the fidelity of translation by verifying mRNA−tRNA complementarity. The 50S subunit contains the site that catalyzes peptide bond formation, namely, the peptidyl transferase center (PTC), which is composed entirely of RNA, making the ribosome a ribozyme. The large subunit also contains a tunnel whose walls are composed mainly of 23S RNA, which protects the growing polypeptide as it exits.

Translation is a multistage process involving, apart from mRNA and tRNAs, many external proteins such as initiation, elongation, and release factors. In general, peptide synthesis can be inhibited at any of these stages of bacterial translation by binding of the inhibitor to either external factors or to sites on the ribosome.[4] Inhibition of peptide synthesis in bacteria can also be achieved by targeting RNA motifs on the ribosome.[2,3,5] Conventional antibiotics bind to specific sites (mainly rRNA) both on the 30S and 50S subunit in bacteria.

There are a number of known antibiotic binding sites in the 30S subunit as shown in Figure 1. For example, paromomycin interferes with decoding and induces misreading, and hygromycin B inhibits translocation of tRNAs, whereas tetracycline inhibits the accommodation of tRNA by binding to the site overlapping with the stem-loop of aminoacyl-tRNA.[1,3] The locations of the antibiotic binding sites in the 30S subunit are spread over 16S RNA (Figure 1), contrary to the binding sites of most known agents that inhibit the function of the 50S subunit, which are concentrated in the PTC or at the entrance to the peptide exit tunnel.[1] These closely positioned agents are macrolides, lincosamides, streptogramines, oxazolidinones, chloramphenicols and puromycin. Puromycin mimics the aminoacylated end of the aminoacyl-tRNA, but its non-hydrolyzable amide bond cannot be cleaved. Erythromycin, a macrolide, binds to 23S RNA at the entrance of the peptide exit tunnel and aborts peptide growth by restricting its egress from the 50S subunit. Oxazolidinones (with linezolid as a representative) are synthetic antibiotics that bind to PTC in

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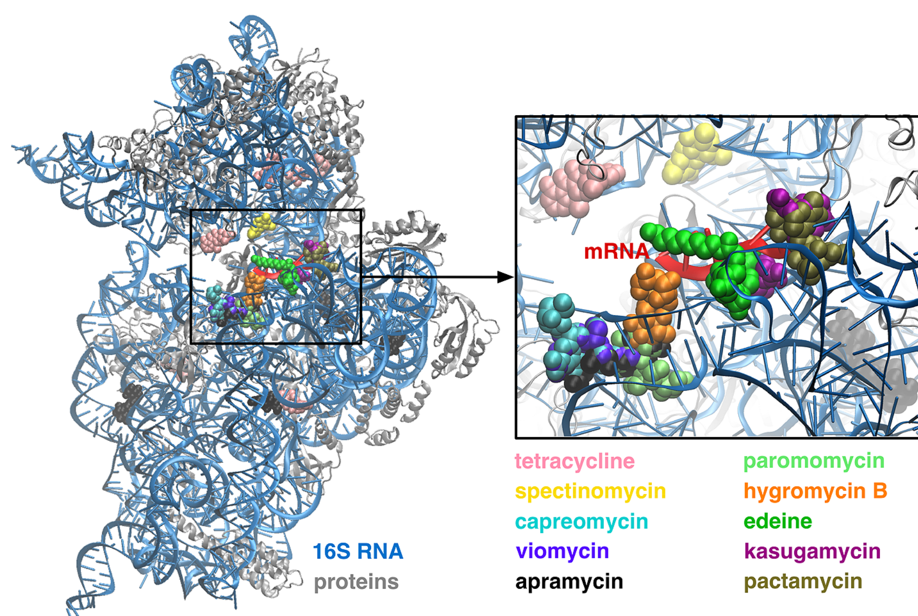

Figure 1. Positions of antibiotics superimposed on the 30S subunit of *T. thermophilus*. Antibiotics are shown as van der Waals spheres with PDB codes: paromomycin, 1IBK;[7] spectinomycin, 1FJG;[8] pactamycin, 1HNX;[9] hygromycin B, 1HNZ;[9] edeine, 1I95;[10] capreomycin, 3KNN;[11] viomycin, 3KNJ;[11] kasugamycin, 2HHH;[12] tetracycline, 1I97;[10] apramycin, 4AQY.[13] The latter three crystallized in many binding sites. Some of the sites overlap.

the A-site cavity of 23S RNA. In contrast, thiopeptides and girodazole bind to distinct sites of the 50S subunit compared to other antibiotics. There have been many reviews on the mechanisms of action and binding sites of conventional antibiotics targeting the ribosome, which give the mechanism of action and describe the binding site of each antibiotic, e.g., refs 1, 3, 5, and 6.

The drawbacks of current antibiotics are their possible toxic side effects, emerging bacterial resistance, and lack of specificity toward the ribosomes of pathogenic bacteria. Bacteria have developed many resistance mechanisms against anti-bacterial compounds targeting their ribosomes. They produce enzymes that methylate the rRNA or chemically modify antibiotics, which reduces antibiotic binding affinity. Other resistance mechanisms include actively extruding or preventing the antibiotic entry or altering the target by mutations of rRNA. Therefore, new anti-bacterial agents are constantly needed, preferably such that make it difficult for bacteria to develop resistance. Due to relatively slow progress in identifying new classes of antibiotics, novel ideas should be investigated. Designing new scaffolds or modifying known compounds has been of moderate success and most importantly will not eliminate cross-resistance.

Here, we will summarize some approaches that are used when looking for new antibiotics and targets. Ribosomal sequences and in particular rRNA, being more evolutionarily stable than proteins, make an ideal target. We will also describe the compounds that have been proven to have some inhibitory activity against the ribosomes; these are natural and synthetic oligonucleotides and peptides.

■ APPROACHES USED TO IDENTIFY OR DESIGN NEW ANTIBIOTICS

Identification of Antibiotics According to Biological or Binding Activity. In the search for new anti-bacterials, the classic approach used is to screen potential compounds for their ability to inhibit growth of whole bacteria, using minimal inhibitory concentration (MIC) as a standardized measure of effectiveness. However, this approach could unintentionally omit promising compounds against certain organisms because these screens typically use a group of representative bacterial strains, which depends on the application.[14] In addition, this approach is also not suitable for identifying inhibitors of a particular process such as bacterial translation for which more specific assays are necessary.

Several *in vitro* assays have been developed for identification of inhibitors of transcription/translation. All of these employ bacterial cell extracts that contain the machinery needed to carry out protein synthesis, supplemented with a reporter gene, typically encoding luciferase.[15−17] The concentration of the inhibitor required to reduce synthesis of reporter protein by 50% (IC_{50}) is used as an indicator of effectiveness. The advantage of using such an assay is that since there is no cell wall barrier to overcome, it is more sensitive than MIC assays, requiring less inhibitor. Moreover it can be used for high throughput screening, and the results may be obtained within a few hours. Several compounds were identified as translation inhibitors by this method.[18−20] However, it is important to use suitable cell extracts for the assays, because the IC_{50} of an inhibitor against the cell extract of a particular bacteria is the best predictor of the MIC against only that particular bacteria rather than of any others.[17] The drawback of this screening method is that one does not know the stage of protein synthesis that is affected. After potential compounds have been identified, it is necessary to characterize them further to determine their target. If the ribosome is inhibited, the exact rRNA site that is targeted by the inhibitor and its mode of action needs to be determined.[21,22] Finally, the inhibitor needs to be tested on whole bacteria.

If an inhibitor against a specific rRNA target site needs to be identified, it is possible to perform binding assays against these isolated targets. First, it is necessary to validate the structure of

the RNA fragment to confirm that it adopts a conformation similar to that of the corresponding region in the ribosome. An example of an RNA fragment used to screen for ligands is the decoding center (30S subunit A site in Figure 2). The binding of the ligand may be monitored using different methods including isothermal titration calorimetry (ITC) and Förster resonance energy transfer (FRET), where chosen mobile bases of the RNA[23,24] or a competing ligand[25] have fluorescent labels. This method has been validated with known inhibitors of the A site[25] and was used to confirm the ability of a computationally designed class of molecules to bind and inhibit the target.[26]

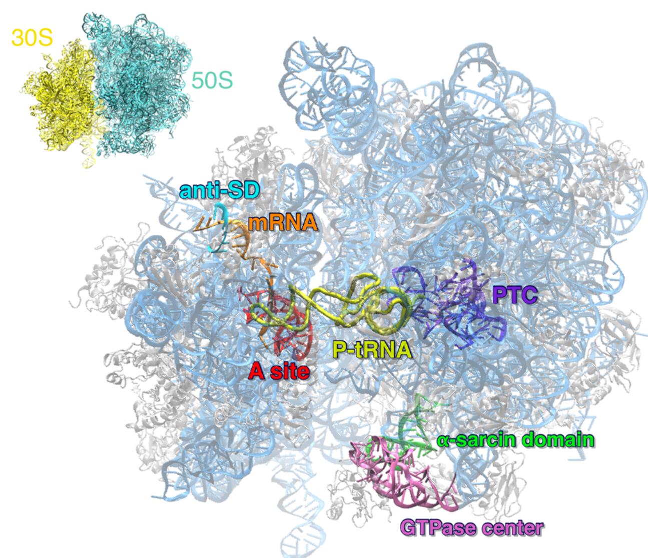

Figure 2. Structure of the *E. coli* 70S ribosome (PDB codes 3R8T and 4GD2;[35] proteins are in silver, RNA in light blue, the L9 protein was removed.) The inset shows the division into subunits.

The application of phage-display technology to specifically identify peptides that target RNA is relatively new. Phage display uses a library of bacteriophages that have a diverse range of unique peptides on their surface.[27] The peptides that bind to the RNA fragment can be affinity-purified together with the phage attached to it, and then the gene sequenced to identify the peptide. This technology has been used successfully to find peptides that bind to the A site,[21] helix 18 of 16S rRNA,[22] and helix 31 of 16S rRNA.[28] Moreover some of these peptides were found to have low dissociation constants, down to 1.1 μM bound to 30S subunits,[22] and even inhibited bacterial translation *in vitro*.[21,22,28] However, occasionally there are problems with insolubility of the selected peptides so they cannot be used for further tests.[22] Targeting rRNA with peptides also has drawbacks because natural peptides are susceptible to protease degradation, but this may be overcome by designing peptide mimetics.

The above approach of screening for binders using RNA fragments generally utilizes only known inhibitor binding sites in rRNA. To take the search for new antibiotics further, it is necessary to examine new targets. The complex ribosome machinery offers many potential sites for interference of function. A widely used method to identify functionally important bases is to introduce mutations at those sites in rRNA and examine if they have a deleterious effect on translation.[29−32] There appears to be a correlation between the position of deleterious mutations in rRNA and known antibiotic binding sites; a few unexplored sites have also been identified.[30,32]

The above methods rely at least partially on random screening of compounds, each with its pros and cons. While screening for antibiotics using whole bacteria has the advantage of only identifying compounds that are able to enter the cell, none of these methods is able to predict the spectrum of action until they are tested, and promising compounds cannot be rationally optimized until studied further to determine their structure and mode of action.

Structure-Based Computational Methods Used To Design Anti-ribosomal Compounds. Thanks to structural biology techniques, many crystal structures of bacterial ribosomes in the free state and in complexes with antibiotics have been solved, e.g., refs 7−13 and 33−35. Also, X-ray and NMR structures of some model RNA constructs (such as the A-site of the 30S subunit) in complexes with antibiotics are available.[36] These atomic-resolution views of ribosomes suggested mechanisms of antibiotic actions and enabled the structure-based design of compounds targeting rRNA (reviewed in refs 37−39). Such design decreases the cost by selecting and ranking the most promising molecules, which reduces the numbers of compounds to be synthesized and checked for *in vivo* inhibition. In general, the approaches to search for compounds targeting rRNA involve improving the existing ribosome inhibitors or designing new scaffolds for both known and yet unexplored binding sites. Derivatization of known compounds to increase their binding affinities and avoid resistance through chemical modifications by bacterial enzymes is the most common way to rationally design antibiotics.

The computational techniques allow for virtual screening of chemical databases of compounds, docking selected compounds to determine their binding mode in the target, and optimizing the lead compounds. The conformations of the antibiotics in the ribosome structures help to design pharmacophoric points (steric and electronic features of a ligand necessary to achieve specific interactions with the receptor), which are used for screening of compound databases. Also, the ribosome structures enabled docking by supplying the geometry of the rRNA target. Docking involves optimizing the position of the ligand in the target site by scoring the conformations of the complex based on the molecular mechanics potential energy function. Other techniques such as molecular[40] and Brownian[41] dynamics and Poisson−Boltzmann electrostatics[42,43] have been also applied thanks to the atomic resolution ribosome structures (reviewed in refs 44 and 45).

The rational and systematic design of small molecules targeting rRNA has seen some progress. Novel oxazolidinones targeting the 50S subunit are in clinical trials.[46] Also, the derivatives of tetracycline (such as omadacycline), aminoglycoside sisomicin (plazomicin), and a macrolide solithromycin have been in development.[46] Ribosome structures suggested the design of tandem molecules, bridging the antibiotics with overlapping or adjacent sites such as linezolid and sparsomycin[47] and aminoglycosides, paromomycin and hygromycin B.[48] In the latter case even though the hybrid ligands were active as inhibitors of bacterial translation, they were not more potent than natural aminoglycosides. Computational screening has also been applied to design new scaffolds targeting the decoding site.[25,49]

Nevertheless, the computer-aided approach for anti-ribosomal binders has yet to show spectacular successes; the reasons

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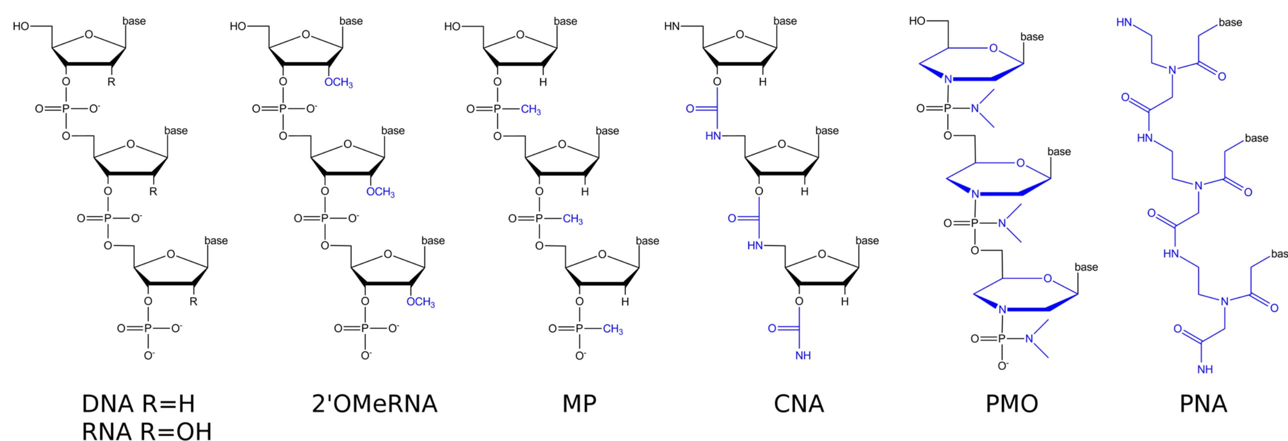

Figure 3. Examples of modified oligonucleotides with the differences from natural ones marked in blue. 2′OMeRNA stands for 2′O-methyl-RNA, MP for DNA methylphosphonates, CNA for methylcarbamate DNA, PMO for phosphorodiamidate morpholino oligomer, and PNA for peptide nucleic acid.

are manifold. These methods have been previously parametrized for proteins, and the development of the potential energy functions with parameters used to design and score RNA-targeting compounds have lagged behind. Numerous features of nucleic acids, and especially of RNA, make the structure-based methods difficult for these systems. First, RNA has a hierarchical structure, usually nonglobular with many tertiary motifs, which results in large intrinsic RNA flexibility. Adaptive binding and induced fit, difficult to account for in the docking procedures, can be significant in RNA so in order to reliably describe the RNA–ligand recognition one needs to account for the internal dynamics of RNA. Second, the high charge of the RNA backbone requires careful treatment of electrostatics. The fixed point-charge per atom models do not account for high polarizability of the phosphate groups. Third, water molecules and ions, which are pivotal for maintaining the correct RNA tertiary structure, can also bridge the RNA interactions with ligands or be displaced upon binding. Therefore, they often need to be positioned explicitly and well equilibrated in the simulation. Next, divalent cations, such as Mg^{2+}, are frequently tightly bound to RNA, assisting in folding and catalysis and affecting RNA mobility. The typical all-atomic mechanical models of RNA with the potential energy formula and parameters are still under development.[50,51]

Predicting the binding mode of any ligand to RNA (the docking problem) is difficult, especially for charged antibiotics. It involves searching and scoring of conformations of the ligand in the binding site of the receptor. Accurate methods assume that both the ligand and receptor are flexible, but to make the procedure computationally tractable approximations must be included. The docking methodologies toward rRNA targets have been verified.[52−55] For charged aminoglycosides the standard protocols did not correctly reproduce their binding modes.[54,55] To improve the performance the authors had to modify the scoring functions to account for the bridging waters and incorporate the flexibility of 16S rRNA. These studies show that the scoring functions for RNA–ligand interactions need to be redesigned to properly capture the balance between the charged and nonpolar interactions.[50,52,54]

Sequence-Specific Inhibition of rRNA by Oligonucleotides. Since many anti-bacterials that work by inhibiting bacterial translation are rRNA binders, in principle, rRNA function can also be inhibited by antisense oligonucleotides that bind in a sequence-specific manner, observing Watson–Crick base pairing. Typically antisense oligonucleotides (binding to RNA) that have been used as anti-bacterials have been targeted against mRNA encoding essential proteins.[56] Nevertheless since the 1970s, antisense molecules, particularly DNA, complementary to exposed regions of rRNA, have been used to study ribosomes. They have been useful in determining accessibility maps of rRNA,[57−59] quaternary structure,[60−62] and functional regions of the ribosome.[63] As a consequence of these functional studies, it has been established that oligonucleotides are capable of inhibiting ribosome function by acting as steric hindrances.

One of the first ribosomal targets identified for antisense inhibition was the anti-Shine–Dalgarno (anti-SD) region (Figure 2). This sequence is located at the 3′ terminal region of 16S RNA, is complementary to the SD sequence found upstream of the start codon in bacterial mRNA, and is important for the initiation of protein synthesis by positioning the start codon at the P site in the ribosome. An oligonucleotide complementary to the anti-SD sequence could be a particularly effective inhibitor by interfering with the binding of mRNA to the ribosome.[64−66] Indeed, Taniguchi and Weissmann showed that a ribooligouncleotide with eight bases complementary to the anti-SD region inhibited formation of the initiation complex.[64] A similar effect was observed when a pentanucleotide composed of a mixture of RNA and DNA bases was used.[65] Some other functionally active regions that have been targets of successful inhibition by oligonucleotides are the A site in 16S rRNA,[67] α-sarcin loop,[68−70] and PTC[69] located in 23S rRNA (for the location of these sites in the ribosome see Figure 2). Therefore theoretically, on condition that a suitable functionally important region is chosen, this simple hybridization concept can be used to search for sequence-specific inhibitors.

To achieve the desired inhibitory effect on bacteria, oligonucleotides need to have high target affinity and metabolic stability. Natural oligonucleotides undergo rapid degradation by cellular nucleases. Therefore various modified oligonucleotides have been designed to introduce features that give them different advantages and effects; either linkages between the bases or the structure of the sugar ring have been modified. Some modifications change the overall net charge of the oligomer and its hydrophobicity. Examples of modified oligonucleotides are presented in Figure 3. Some of these have been successfully used for inhibition of the bacterial

ribosome. Their individual properties will be described in their respective sections.

DNA and Its Modifications. DNA methylphosphonates (MP) are nonionic DNA analogues in which a nonbridging oxygen atom in the phosphate group is replaced with a methyl group. MPs, complementary to the anti-SD region (Figure 2), inhibited translation *in vitro* if they were at least 6 nucleotides in length.[66] These sequences were even able to inhibit colony formation of a permeable strain of *E. coli* (inhibition of up to 97% at 75 μM); however, their size prevents their entry into wild-type bacteria. Methylcarbamate DNA (CNA) analogues also have the ability to inhibit translation *in vitro* as well as in the permeable *E. coli* strain. However while these oligonucleotides can form stable duplexes with RNA, the rigidity of their backbone limits their functional length. As a result, shorter sequences were found to be more effective. Their conjugates with poly(ethylene glycol) increased their solubility and also allowed entry into normal *E. coli*, exhibiting 75% inhibition at 180–200 μM.[71]

The α-sarcin loop, shown in Figure 2, is another promising rRNA target shown. It interacts with elongation factors and is located deep in the ribosome structure, and yet antisense DNA could bind and inhibit its activity *in vitro*.[68] However, later studies have shown the importance of the oligonucleotide design including length and position. A set of DNA sequences against the α-sarcin loop showed that the oligonucleotide with the highest sequence coverage had the greatest inhibitory activity, with a 2.3-fold increase observed when the length was increased from 11 to 15 nucleotides.[72] It has been demonstrated that DNA against the α-sarcin loop can be designed to be highly specific against selected organisms (e.g., Mycobacteria) by using subtle differences in the rRNA sequence, provided that functionally important residues as well as residues that make the oligonucleotide specific are blocked.[70]

The GTPase-associated center of the ribosome (Figure 2) interacts with translational GTPases such as elongation and release factors. Its 23S RNA part is known to be a good target for oligonucleotides because it is accessible and mutational studies of bases in this region have been able to inhibit interaction of the ribosome with release factor 2.[73] DNA sequences against this site had the ability to inhibit translation *in vitro* to varying degrees, with shorter sequences appearing to have the advantage of being able to access the target more easily.[72]

Often, the metric of success used is the comparison of oligonucleotide inhibition to antibiotics that are known to inhibit the ribosome, such as tetracycline. It is a useful control to include because the results of experiments performed on different *in vitro* systems are difficult to compare due to variability of cell extracts or cell number. Since such a control was not included in the above examples, it is only possible to compare inhibition by these oligonucleotides with those tested in the same study.

PNA. A lot of work on sequence-specific targeting of rRNA has been done with the use of peptide nucleic acids (PNAs). A PNA is a DNA mimic with a backbone composed of repeating *N*-(2-aminoethyl) glycine units linked by amide bonds with organic bases attached to the central amine through methylene carbonyl linkages[74] (Figure 3). The neutral backbone increases the affinity of a PNA toward natural nucleic acids because there is no interstrand electrostatic repulsion of the phosphate backbones. Thus PNA/DNA or PNA/RNA duplexes have higher thermal stability compared to duplexes of natural nucleic acids. Furthermore PNA oligomers containing solely pyrimidine bases have the ability to hybridize to complementary double-stranded DNA through strand displacement.[75] Bis-PNA, which is a structure consisting of two PNA strands connected via a neutral flexible linker, can invade natural double-stranded nucleic acids and form triplexes via Watson–Crick and Hoogsteen base pairing.[76] To ensure pH independence of binding of the Hoogsteen strand, the cytosine is often replaced by pseudoisocytosine (J).[77] In addition to high binding affinity, PNA possesses strong sequence discrimination ability compared to DNA,[75,77,78] and even one base pair mismatch can lead to significantly decreased melting temperature,[79] making it highly specific. Moreover a PNA's synthetic structure results in its high resistance to nucleases and proteases,[80] which together with low toxicity to eukaryotic cells[81] makes PNA a good candidate for a potential antibiotic.

Studies of PNAs targeting functional domains of both ribosomal subunits have demonstrated that duplex-forming PNA can inhibit neither bacterial growth nor protein synthesis in a cell-free (*in vitro*) translation system[69] unless it is attached to a cell wall-permeabilizing peptide such as $(KFF)_3K$, which is proved to improve cell entry of the anti-ribosomal PNA oligomers. Hatamoto and colleagues designed a duplex-forming PNA-peptide conjugate targeting the anti-SD site on 16S rRNA that inhibited translation *in vitro* (IC_{50} of 0.6 μM) as well as bacterial growth of *E. coli* wild-type strain K12 in a sequence-dependent manner (MIC of 10 μM).[82]

However it seems that it is triplex-forming bis-PNA that is the most potent against rRNA functional domains. Good and Nielsen designed effective bis-PNAs targeting PTC and the α-sarcin loop domain at the 23S rRNA[69] (Figure 2). Their inhibitory effect on *in vitro* translation was similar to that of tetracycline. These two bis-PNA sequences also inhibited bacterial growth of an *E. coli* cell-wall-permeable strain AS19 (in 10 times diluted lysogeny broth (LB), unlike standard procedure) with an IC_{50} of 2 μM and 5 μM against the PTC and the α-sarcin loop, respectively, compared to an IC_{50} of 0.1 μM for the tetracycline. The same PNA sequence against the α-sarcin loop was later examined by Good et al.[81] as a conjugate with $(KFF)_3K$ peptide and proved to even inhibit growth of *E. coli* wild-type strain K12 in full-strength Mueller Hinton broth, showing a MIC of 3 μM, while in 10 times diluted broth the MIC was 0.7 μM. It is unfortunately hard to deduce whether or how much the attachment of the peptide to bis-PNA improved the inhibitory effect *in vitro* since the conditions of the two experiments were different and no results for tetracycline were indicated in the second paper.

Similarly Xue-Wen et al. designed bis-PNAs and their conjugates with the $(KFF)_3K$ peptide targeting different sites associated with the GTPase center in 23S RNA[83] (Figure 2). In the cell-free translation system the best working bis-PNA oligomer covering the G1138 nucleobase had an IC_{50} of 0.15 μM, comparable to that of tetracycline. For this bis-PNA conjugated with the peptide and tested on *E. coli* DH5α strain (in 10% strength LB) the MIC was 10 μM, 2.5 times higher than that of tetracycline. The unconjugated bis-PNA showed no growth inhibition of bacteria up to 50 μM, which again proved that conjugation with the peptide is necessary for PNA to inhibit bacterial cells. However it has not been shown whether PNA–peptide conjugate had any effect on cell-free translation inhibition.

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2′OMe RNA. The RNA analogue, 2′O-methyl-RNA is methylated at the 2′ position of the ribose sugar, which ensures better biostability against nucleases in comparison with naturally occurring nucleic acids as well as enhanced binding affinity for RNA[84] (Figure 3). 2′OMe oligomers show reduction in general toxicity,[85] but similar to PNA they do not induce RNase H activity (RNase H is an endonuclease with hydrolytic activity toward RNA in DNA/RNA duplexes). 2′OMe oligonucleotides however were found to inhibit *in vitro* translation. Abelian et al. designed several overlapping 10-mer 2′OMe oligonucleotides complementary to the A site of 16S rRNA (Figure 2) in the region spanning A1485 to C1510.[67] Two of these oligomers targeting the region A1493−G1504 bound to the targeted rRNA strand strongly; the best covering A1493−A1502 showed the lowest K_d, 29 nM. The authors showed that antibiotics that also bind to the A-site and were expected to compete did not inhibit the binding of these oligomers. Moreover, the aminoglycosidic antibiotic paromomycin enhanced the binding affinity of the A1493 oligomer 7.5-fold. This is probably due to paromomycin driven displacement of bases A1492 and A1493, which makes those bases available for favorable interactions of rRNA with oligonucleotides. The binding affinities correlated with *in vitro* translation inhibition properties of the 2′OMe RNA oligomers. The two oligomers with highest inhibition (A1493 and A1499) had IC_{50} values of about 10 μM, in the same range as antibiotics targeting this region, paromomycin (IC_{50} of 10 μM) and hygromycin B (IC_{50} of 50 μM).

PMO. Phosphorodiamidate morpholino oligomer (PMO) was designed to block translation or gene expression through steric hindrance in a sequence-specific manner.[86] PMO is a DNA analogue with the sugar ring replaced by a morpholine group and the phosphodiester substituted by phosphorodiamidate linkage (Figure 3). These alternations result in an uncharged backbone unable to induce RNase H activity, high affinity and specificity for complementary targets,[86] and complete resistance to nucleases.[87] PMOs, like other nucleic acid analogues, are inefficiently taken into bacterial cells and require special delivery to increase the uptake. Therefore experiments conducted on bacteria required conjugating PMO to the cell penetrating peptides such as $(KFF)_3K$, as mentioned previously, or others. Mellbye et al. studied PMOs targeting the mRNA of the *acpP* gene in *E. coli*, encoding the acyl carrier protein important for lipid biosynthesis, conjugated with different peptides.[88] One of the most potent was $(RXR)_4XB$−PMO (composed of β-alanine (B) and 6-aminohexanoic acid (X)) shown to inhibit bacterial growth in a sequence-dependent manner with a MIC of 1.25 μM, which was five times lower than the MIC of ampicillin. The same PMO−peptide conjugate was also able to reduce bacterial infection in mice and promote survival through a dose-dependent response.

Applications of PMOs to targeting bacterial rRNA are not numerous.[89,90] Geller et al. designed a PMO targeting bases 446−466 of 16S rRNA.[89] This PMO did not inhibit *E. coli* growth at tested concentrations of up to 50 μM but was not conjugated to any cell-wall-penetrating peptide. Geller et al. have shown that PMOs could enter the cell only if the experiments were conducted on *E. coli* mutant SM101 with a defective outer membrane or when covalently attached to the $(KFF)_3KC$ peptide.[89] Other PMO sequences targeting 16S or 23S rRNA have also been tested on other bacterial species.[90]

■ CONCLUSION

As a result of emerging bacterial resistance to known antibiotics, new ligands targeting various bacterial metabolic pathways are needed, but searching for novel compounds using conventional strategies is unlikely to keep pace with acquired resistance. We need to look for other mechanisms of inhibitory activity and expand beyond the currently available classes of antibiotics as well as identify new targets. The bacterial ribosome, in addition to being a known antibiotic target, has unexplored functional sites that may act as targets. Some strategies that may be used to take advantage of these untested sites include using binding assays to identify ligands that interact with rRNA fragments, performing virtual screening of ligand libraries and docking, and designing antisense oligonucleotides that bind to promising sites.

Unfortunately, docking techniques are still not well developed for RNA targets, and therefore ribosome structure-based antibiotic design is difficult. Sequence-specific inhibition of the ribosome is a promising approach for the development of antibiotics. The appropriate choice of the sequence may be used to design highly specific ligands, avoiding not only association with human rRNA but also other, perhaps nonpathogenic, bacteria. However, designing sequences targeting rRNA is not straightforward, since rRNA is large with complicated architecture. Therefore sequence complementarity is not sufficient; one must take into account the accessibility of the target and its tertiary structure. For helical regions, oligonucleotides with good strand-invading properties have to be used. There are also a number of modified nucleotides (e.g., in helix 69 of the 23S rRNA) resulting from post-transcriptional modifications of rRNA that may limit the effective hybridization.

Another issue is the solubility of hydrophobic modified oligomers, although there are ways this may be overcome. For example, the solubility of PNA can be improved by conjugation to cationic groups such as polyamines or lysines.

For an anti-bacterial agent to work it must penetrate into the targeted cells. The major limiting factor in potential applications of antisense anti-ribosomal strategies to treat bacterial diseases is their poor uptake by bacterial cells since oligonucleotides do not readily pass through the bacterial cell wall. Natural antibiotics enter bacteria through various mechanisms that have been developed by evolution. Therefore, conjugation of oligomers with conventional antibiotics may be a promising strategy to achieve cellular uptake. For PNA and PMO the permeability problem has been solved by conjugating them to cell-penetrating peptides. As mentioned previously, a successful peptide whose conjugation to PNAs allowed their use in both Gram-negative and Gram-positive bacteria is $(KFF)_3K$,[81,91] while *E. coli* growth has been inhibited by conjugates of antisense PMO with the peptide $(RXR)_4XB$.[88]

There have been studies performed as proof that oligonucleotides such as PNA and PMO can be used to to treat bacterial infections in animals. These have targeted the mRNA of an essential gene *acpP*, encoding acyl carrier protein, and were shown to decrease bacterial count and/or mortality in mice.[92−94] The conjugate of a PMO with $(RFF)_3RXB$ peptide had the ability to maintain 100% survival at a dose of 30 μg and showed a 15-fold potency compared to ampicillin.[94]

Thus, it is encouraging to know that it is possible to use antisense oligonucleotides *in vivo* as therapeutic agents against bacteria. To apply this strategy efficiently, it is necessary to

identify novel targets for inhibition of bacteria. The field of sequence-specific targeting of rRNA is still developing and has not been explored in detail, with only a few rRNA target sites checked for translation inhibition. This opens room for further studies to search for both new rRNA targets and better-behaved non-natural oligomers that would show better binding and metabolic properties.

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Notes

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KEYWORDS

antibiotics: chemical substances capable of destroying or inhibiting the growth of bacteria and other microorganisms
antisense therapy: the therapeutic use of compounds that bind and block RNA function
bacterial ribosome: the RNA-protein complex in bacterial cells essential for protein synthesis
hybridization: the interaction of complementary strands of nucleic acids through hydrogen bonding of the bases
oligonucleotides: short nucleic acid strands that may be natural or synthetic
RNA: ribonucleic acid
screening: the testing of several, very often random, compounds for desirable characteristics
translation inhibition: inhibition of protein synthesis by blocking function of the ribosome, mRNA or translation factors

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