REVIEW

Antibiotic drugs targeting bacterial RNAs

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Abstract  RNAs have diverse structures that include bulges and internal loops able to form tertiary contacts or serve as ligand binding sites. The recent increase in structural and functional information related to RNAs has put them in the limelight as a drug target for small molecule therapy. In addition, the recognition of the marked difference between prokaryotic and eukaryotic rRNA has led to the development of antibiotics that specifically target bacterial rRNA, reduce protein translation and thereby inhibit bacterial growth. To facilitate the development of new antibiotics targeting RNA, we here review the literature concerning such antibiotics, mRNA, riboswitch and tRNA and the key methodologies used for their screening.

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1. Introduction

Deoxyribonucleic acid (DNA) is the major genetic material in eukaryotes and generally exists in the form of double-stranded helices. In contrast, ribonucleic acid (RNA) can fold into numberless tertiary structures that reflect its diverse functions. Thus it serves as the genetic material in some viruses, as the mediator of genetic information from DNA to protein, as the structural component in many ribonucleoproteins (RNPs) and, in some cases, as a catalyst. RNA is usually associated with RNA-binding proteins (RBPs) which serve to either protect, stabilize or transport it and regulate its interaction with other molecules. RNA plays many crucial roles in protein synthesis, transcriptional regulation and retroviral replication that make it a prime target for drug action.

The recent publication of high resolution crystal structures of prokaryotic rRNA subunits has transformed our understanding of RNA. The structures of RNA alone and of RNA–protein complexes reveal a variety of tertiary structures and patterns of RNA–protein interaction. RNA can fold into complex three-dimensional structures comprising loops, pseudoknots, bulges and turns which afford specific binding sites for small molecules. Compared to DNA, RNA is not only more flexible but lacks repair mechanisms which enhance its susceptibility to the action of therapeutics. These include both natural and synthetic compounds that can influence the biological activity of RNA by changing its configuration or inhibiting its catalytic function.

Many antibiotics are known to target rRNA in prokaryotes and thereby alter protein translation. Their diverse structures (Fig. A and B) witness both to the importance attached to the application of X-ray crystallography which has elucidated many RNA–protein subunits of which approximately two-thirds are RNA. The bacterial 30S subunit near the A-site of the 30S subunit leads to a decrease in translational accuracy and inhibition of the translocation of the ribosome. Binding to the 16S rRNA has been demonstrated by NMR, mass spectroscopy, surface plasmon resonance and X-ray crystallography.

The therapeutic and adverse effects of the aminoglycosides have been intensively studied. The main issue in clinical practice relates to their toxicity and the rapid increase in the emergence of resistant strains. Hopefully, modification and reconstruction of sugar moieties will lead to new aminoglycoside derivatives that will overcome the undesirable properties of the naturally occurring compounds. A small library of four aminoglycosides that bind to a 16384-member bacterial rRNA A-site-like internal loop has been established to recognize the RNA motifs using two-dimensional combinatorial screening (2DCS). This may enable the rational and modular design of small molecules targeting RNA.

2. Antibiotic drugs targeting rRNA

rRNA is the most commonly exploited RNA target for small molecules. The bacterial ribosome comprises 30S and 50S ribonucleoprotein subunits, contains a number of binding sites for known antibiotics and is an attractive target for novel antibacterial agents. The large difference between prokaryotic and eucaryotic rRNA enables rRNA-targeting against a broad spectrum of pathogenic bacteria. Bacterial ribosomes have two ribonucleoprotein subunits of which approximately two-thirds are RNA. The bacterial rRNA includes 5S, 16S and 23S rRNA, the smallest (5S rRNA) being a ~120 nt RNA. The smaller 30S subunit contains a single ~1500 nt RNA (16S rRNA) and about 20 different proteins while the larger 50S subunit contains a ~2900 nt RNA (23S rRNA) and about 30 different proteins. Recently, the application of X-ray crystallography has elucidated many antibiotic-binding sites on the ribosomal subunit facilitating the design of novel antibiotics.

2.1. Aminoglycoside antibiotics

Aminoglycosides are a group of well-known antibiotics that have been used successfully for over half a century. Streptomycin and spectinomycin are typical examples which function by binding to specific sites on prokaryotic rRNA and affecting the fidelity of protein synthesis. The rRNA aminoacyl–tRNA site is a major target for aminoglycosides which, because of the difference between prokaryotic 16S and human 18S rRNA, selectively kills bacterial cells. Binding of drug to the 16S subunit near the A-site of the 30S subunit leads to a decrease in translational accuracy and inhibition of the translocation of the ribosome. Direct binding to 16S rRNA has been demonstrated by NMR, mass spectroscopy, surface plasmon resonance and X-ray crystallography.

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2.1.1. Streptomycin

Streptomycin disturbs several steps of protein synthesis leading to translational errors and slowdown of translocation. It binds firmly to a single site on 16S rRNA with binding to the ribonucleoprotein as supported by footprinting and mutation studies. Footprinting studies showed that streptomycin protects specific residues of 16S rRNA within the 30S subunit and can be linked to specific portions of 16S rRNA. Moreover, a mutation in Euglena chloroplast 16S rRNA resulted in streptomycin resistance and mutations in different regions of Escherichia coli 16S rRNA changed the ribosomal response to streptomycin. Streptomycin also interacts with ribosomal proteins in the 30S subunit and mutations in S4, S5 and S12 ribosomal proteins are shown to influence its binding.

Streptomycin can bind to E. coli 16S rRNA in the absence of ribosomal proteins and can protect bases in the decoding center from dimethyl sulfate (DMS) attack. These interactions were evaluated in the classic high ionic strength buffer (20 mmol/L MgCl2, and 300 mmol/L KCl) used to separate active 30S subunits from 16S rRNA and ribosomal proteins. A similar set of bases was protected by streptomycin in RNA fragments corresponding to 16S rRNA. The magnesium ion is indispensable for the protection of the decoding analog afforded by streptomycin.

2.1.2. Spectinomycin

Spectinomycin is an aminocyclitol antibiotic produced by Streptomyces spectabilis which inhibits the growth of many Gram-negative bacteria and is particularly useful in treating gonorrhea. Chemical footprinting has demonstrated that spectinomycin binds to the N-7 position of E. coli 16S rRNA and the fact that several mutations in RNA and protein lead to spectinomycin resistance implicates a probable binding site in 16S rRNA. Such binding may block the attachment of elongation factor G and thereby prevent the translocation of peptidyl-tRNAs from the ribosomal...
A-site to the P-site. The A(aminoacyl)-site close to the 3′-end of 16S rRNA is highly important in the decoding process such that binding of an aminoglycoside leads to erroneous protein synthesis and bacterial death. A set of overlapping, complementary 2′-O-methyl (OMe) 10-mer oligoribonucleotides was used to target the A-site on purified 30S ribosomal subunits from *E. coli* and shown to be almost ideal inhibitors of *in vitro* translation. However, the correlation of inhibition activity with binding strength to the A-site was limited.54

The X-ray crystallographic structure of the complex between spectinomycin and the 30S subunit of *Thermus thermophilus* confirms that the antibiotic-binding site is in the minor groove near the end of helix 34 of 16S rRNA.23 Spectinomycin can form a stable complex with multiple RNA bases via hydrogen bonding

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**Figure 1** The structures of antibiotic drugs (streptomycin, spectinomycin, tetracycline, and puromycin) whose mechanism of action is related to rRNA. A: Streptomycin, spectinomycin and tetracycline target bacterial 16S rRNA; puromycin resembles the 3′ end of the aminoacylated tRNA. B: Lincomycin, clindamycin, and chloramphenicol target bacterial 23S rRNA; mupirocin targets aminoacyl tRNA synthetase.
Figure 2  A: The secondary structures of partial 16S rRNA (numbers indicate nucleotide positions). Nucleotides interacting with spectinomycin, tetracycline and streptomycin are marked with red, green and yellow circles, respectively. B: The secondary structures of partial 23S rRNA. Nucleotides interacting with chloramphenicol, lincomycin and clindamycin, puromycin and tetracycline are marked with purple, green, yellow, and red circles, respectively.
suggesting that other RNA structures may serve as binding sites for spectinomycin either through homology to helix 34 or by different ensembles of interactions. It has been shown that over-expression of 16S rRNA fragments containing helix 34 can induce some resistance to spectinomycin in vivo.

2.2. Tetracycline

Tetracycline inhibits binding of RNA to ribosomes mainly by influencing binding to the A-site although some reports implicate binding of Ac-Phe-tRNA to the P-site. A strong binding site exists on the 30S subunit and a lot of weaker sites on the 30S and 50S subunits. Tetracycline may incorporate mainly into ribosomal proteins since, in the absence of ribonucleoprotein, 16S RNA and the proteins S3, S7, S8, S14 and S19 show high affinity for tetracycline particularly S7.

In addition, using a photocleavable benzophenone derivative of tRNA [3-(4-benzoylphenyl)propionyl-phenylalanine transfer RNA (BP-Phe-tRNA)], the photocleavage of 23S RNA was completely inhibited by tetracycline and tetracycline itself interacted efficiently with the loop V region of 23S RNA. 16S RNA and 23S RNA are targets of tetracycline and activity data from crosslinked subunits has shown that tetracycline crosslinks with 16S RNA at the strong binding site.

2.3. Lincomycin and clindamycin

Lincomycin is a lincosamide antibiotic which, together with its derivative, clindamycin (7-chloro-7-deoxylincomycin), inhibits bacterial growth by preventing peptide bond formation. The two drugs act by targeting the peptidyl transferase loop in domain V of 23S rRNA of the 50S ribosomal subunit which is the site of peptide bond formation. The peptidyl transferase loop has a complex tertiary structure probably containing the adjacent hairpin loop. The fact that a transition mutation at position 2032 leads to clindamycin resistance in E. coli and lincomycin resistance in tobacco chloroplast supports this mechanism of action. The fact that clindamycin is more potent than lincomycin in inhibiting the growth of Gram negative bacteria is probably the result of its higher lipid solubility that enables it to more readily penetrate the bacterial outer membrane and bind at the same ribosomal target site. In vitro chemical footprinting indicates that the two antibiotics interact with 23S rRNA in E. coli ribosomes.

2.4. Chloramphenicol

Chloramphenicol is a broad spectrum antibiotic which acts as a potent inhibitor of bacterial protein biosynthesis. It has a long clinical history but bacterial resistance is common. Chloramphenicol footprinting studies with specific nucleotides has revealed the binding sites to be on the 50S ribosomal subunit where chloramphenicol interacts with the central loop of 23S rRNA domain V to inhibit peptidyl transferase activity. Details of the binding to the 50S subunits in Deinococcus radiodurans and Haloarcula marismortui have been revealed by X-ray studies. Mutations in RNA can affect chloramphenicol binding.

3. The possibility of targeting with bacterial messenger RNA (mRNA)

Some novel drugs to target eukaryotic mRNAs are now being developed. Although the structure of mRNA is less complicated than that of rRNA, it still incorporates some special structures such as hairpins and pseudoknots that provide binding sites for small molecules. For example, the iron response element (IRE) present in several mRNAs involved in iron homeostasis and identified in Alzheimer's amyloid precursor protein is considered to be a target for small molecules. Another is the non-structured AU-rich element that spatiotemporally regulates mRNA translation and stability. Some of these interactions are critical for physiological processes and are being explored as targets for drug discovery.

Certain mRNAs also use allosteric control to mediate regulatory responses. For example, the mRNAs encoding enzymes involved in thiamine (vitamin B1) biosynthesis in E. coli can bind to thiamine or its pyrophosphate derivative without the need for protein cofactors. In addition, bacterial riboswitches that consist of structured RNA domains usually residing at the 5' untranslated region of mRNAs can directly bind specific metabolites and serve as logic gates regulating their own expression without the need for any regulatory proteins. RNA switches may serve as novel targets for drug discovery since they are widely used by bacteria to sense changes in cell physiology and to regulate metabolic pathways. In depth information on this topic is available in the literature.

Notwithstanding the above, there are big differences between prokaryotic and eukaryotic mRNAs. These include: (1) the fact that prokaryotic mRNA does not need to be processed or transported so that translation by the ribosome can begin immediately after the end of transcription or coupled with transcription. Splicing of pre-messenger RNA into mature messenger RNA is an essential step for the expression of most genes in eukaryotes and is being employed for disease therapy; (2) the fact that, in general, the lifespan of prokaryotic mRNA is much shorter than that of eukaryotic mRNA because the absence of a 5' cap and 3' polyA in prokaryotic mRNA enables its immediate degradation by exonuclease.

4. Antibiotics relevant to bacterial tRNA

4.1. Puromycin

Puromycin is an aminonucleoside antibiotic derived from Streptomyces albogriseus which causes premature chain termination during translation in the ribosome. Part of the puromycin molecule resembles the 3' end of the aminoacylated tRNA such that it enters the A-site and transfers to the growing chain leading to an immature puromycylated chain and its premature release. However, the lack of selectivity of puromycin makes it unsuitable as an antibiotic and it is now mainly used as a biochemical tool to study protein synthesis. It is also under investigation as an anticancer drug.

4.2. Mupirocin

Aminoacyl tRNA synthetase (aaRSs) has been recognized as a useful drug target and many natural compounds specifically target it to inhibit bacterial growth. In fact there are some 20 essential aaRSs, each of which may represent a potential target for novel antibiotics. However, mupirocin (pseudomonic acid A) is the only marketed drug that targets this enzyme. Mupirocin exhibits good activity against Coagulase-negative Enterococcus faecium, Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus aureus, Streptococcus pyogenes and Streptococcus pneumoniae.
It has also been shown in in vitro studies to inhibit the growth of pathogenic fungi such as dermatophytes. Recent genomic and biochemical research has provided a wealth of information relevant to aARS including their crystal structures and active sites. Clearly they represent promising targets for novel antibiotics95.

Mupirocin inhibits iso-leucyl-tRNA synthetase (IleRS) and represents the starting point for the development of other aARS inhibitors. Pseudomonas fluorescens has a 74-kb gene cluster encoding mupirocin which includes polyketide synthetase and a fatty acid synthetase system66,67. Mupirocin is a secondary metabolite produced during the late stationary phase68 which inhibits protein synthesis by specifically binding to bacterial IleRS and inhibiting the formation of Ile-tRNA. Selectivity studies have demonstrated that mupirocin inhibits bacterial, archaeal and fungal IleRS but not their mammalian orthologs69. There are two genes (ileRS1 and ileRS2) in P. fluorescens which display remarkable differences100. IleRS2 has no sensitivity to mupirocin and exhibits eukaryotic features suggesting that, in P. fluorescens, the ileRS2 gene functions to protect the bacteria from mupirocin attack100. A mutation in E. coli thrS, a Thr-tRNA ligase, can resist an inhibitor of deacetylase LpxC, one of the most promising targets for the treatment of multidrug-resistant Gram-negative infections101. Structural and energetic aspects of the binding of aristololactam-β-l-glucoside and daunomycin to tRNA(phe) have been investigated using various biophysical techniques102.

5. Techniques crucial for the discovery of drugs targeting RNA

Great strides have been made in the discovery of drugs targeting RNA. High-resolution NMR, electrospray mass spectroscopy (ESI-MS), surface plasmon resonance (SPR), together with other technologies have facilitated discovery in this field. For example, the structure of a 27 nucleotide RNA complexed with an aminoglycoside antibiotic has been determined by NMR spectroscopy70; a technique based on ESI-MS (IBIS Therapeutics) can screen tens of compounds (T766 and T054) with potent specificity against M. tuberculosis and low toxicity to mice and other bacterial strains.

6. The future of antibiotics targeting bacterial RNAs

The genomic revolution has revealed many RNAs as potential targets for novel antibiotic drugs. Targeting RNA is challenging and complementary to traditional drug discovery that focuses on proteins and may have some advantages. First, more sites are accessible at the RNA level while targeting proteins is usually restricted to their active sites. Secondly, it is cost-effective to subject RNAs to high-throughput screening. With developments in new drug discovery technologies, targeting RNAs for better antibiotics is emerging as a new frontier in drug discovery.

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