REVIEW

Antibiotic drugs targeting bacterial RNAs

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Weiling Hong, Jie Zeng, Jianping Xie*

Institute of Modern Biopharmaceuticals, State Key Laboratory Breeding Base of Eco-Environment and Bio-Resource of the Three Gorges Area, Key Laboratory of Ministry of Education Eco-Environment of the Three Gorges Reservoir Region, School of Life Sciences, Southwest University, Chongqing 400715, China

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.

*Corresponding author. Tel./fax: +86 23 68367108.
E-mail addresses: georgex@swu.edu.cn, jianpingxiefudan@gmail.com (Jianping Xie).

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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[1]. 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[2]. 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[3,4]. 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[5–8]. The structures of RNA alone[9,10] and of RNA–protein complexes[11] 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[12]. Compared to DNA, RNA is not only more flexible but lacks repair mechanisms which enhance its susceptibility to the action of therapeutics[13]. These include both natural and synthetic compounds that can influence the biological activity of RNA by changing its configuration or inhibiting its catalytic function[13,14].

Many antibiotics are known to target rRNA in prokaryotes and thereby alter protein translation[15,16]. Their diverse structures (Fig. 1A and B) witness both to the importance attached to this class of drugs and to the long period of their development since their discovery in the 1930s. Concomitant with the marketing of powerful antibiotics has emerged the phenomenon of antibiotic resistance which poses a serious threat to human health. The recent slow-down in the pace of novel antibiotic development[17,18] has further complicated the global health issue. However, antibiotics remain an attractive area for investment and represent the third largest therapeutic area with global financing of more than $34 billion in 2006[19]. To assist in the discovery of new antibiotics, we here summarize the most commonly used drugs targeting bacterial rRNA in clinical development.

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[20]. 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[21]. Recently, the application of X-ray crystallography has elucidated many antibiotic-binding sites on the ribosomal subunit[22,23] facilitating the design of novel antibiotics Fig. 2.

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 (rRNA A-site) is a major target for aminoglycosides which, because of the difference between prokaryotic 16S and human 18S rRNA, selectively kills bacterial cells[24]. 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[23,25–29]. Direct binding to 16S rRNA has been demonstrated by NMR[27], mass spectroscopy[29], surface plasmon resonance[28] and X-ray crystallography[23].

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[30]. 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[31].

2.1.1. Streptomycin

Streptomycin disturbs several steps of protein synthesis leading to translational errors and slowdown of translocation[32–35]. It binds firmly to a single site on 16S rRNA[36–38] without binding to the ribonucleoprotein[39] as supported by footprinting and mutation studies. Footprinting studies showed that streptomycin protects specific residues of 16S rRNA within the 30S subunit[40] and can be linked to specific portions of 16S rRNA[41]. Moreover, a mutation in *Euglena* chloroplast 16S rRNA resulted in streptomycin resistance[42] and mutations in different regions of *Escherichia coli* 16S rRNA changed the ribosomal response to streptomycin[43–48]. Streptomycin also interacts with ribosomal proteins in the 30S subunit[49,50] and mutations in S4, S5 and S12 ribosomal proteins are shown to influence its binding[51].

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[52]. These interactions were evaluated in the classic high ionic strength buffer (20 mmol/L $MgCl_2$, 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[40] and the fact that several mutations in RNA and protein lead to spectinomycin resistance implicates a probable binding site in 16S rRNA[53]. Such binding may block the attachment of elongation factor G and thereby prevent the translocation of peptidyl-tRNAs from the ribosomal

A

Streptomycin

Tetracycline

Spectinomycin

Puromycin

B

Lincomycin

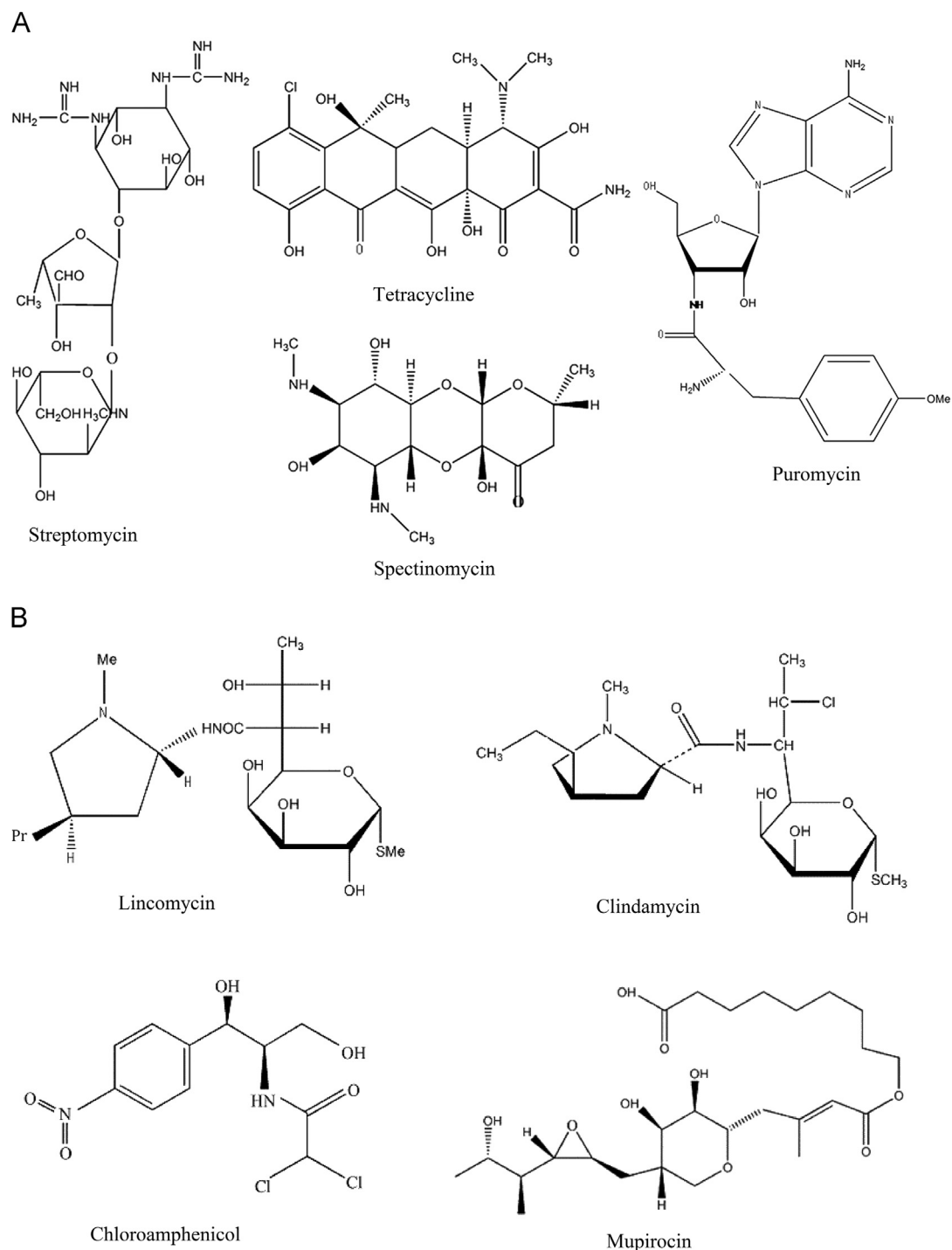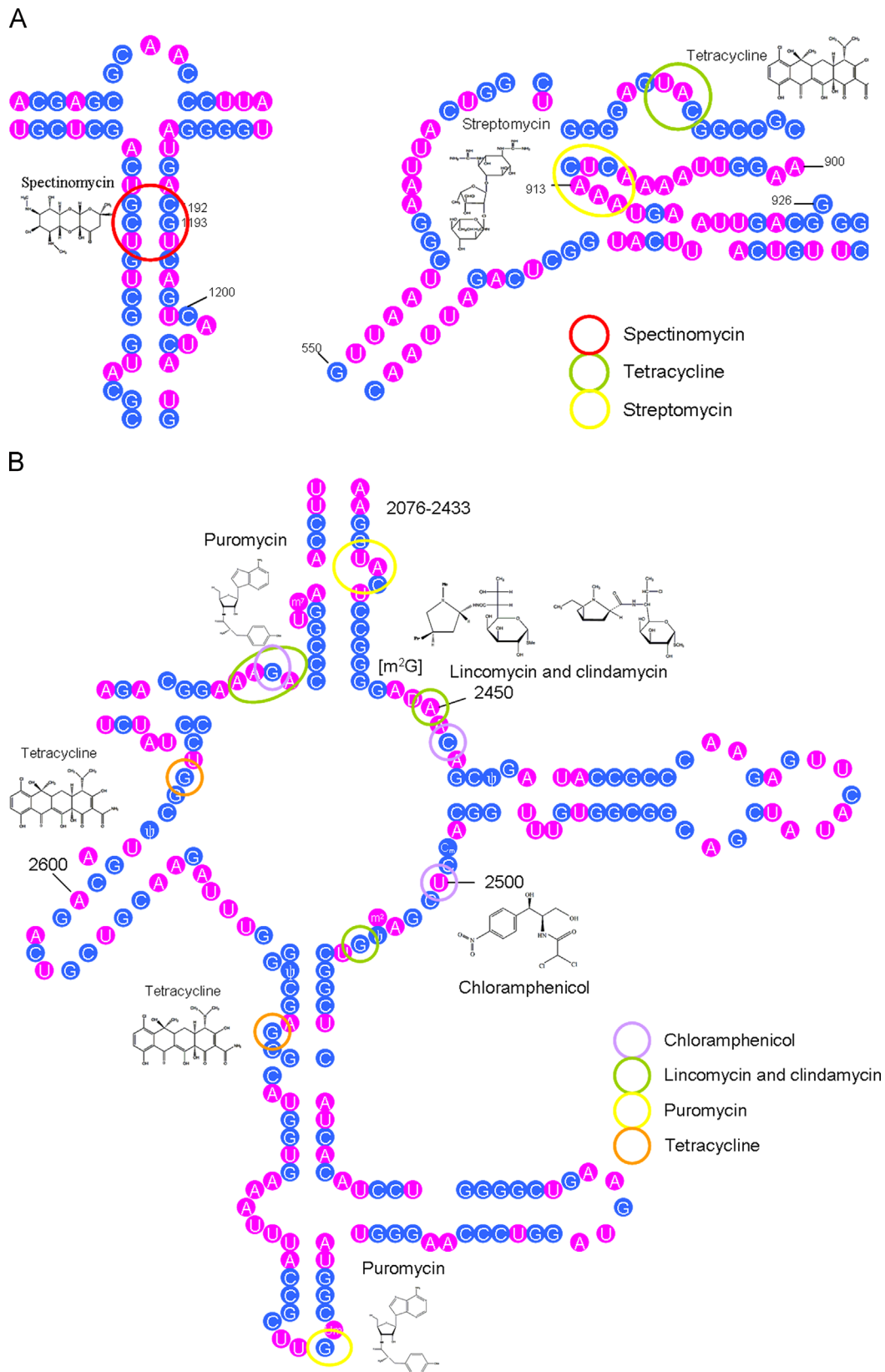Clindamycin

Chloroamphenicol

Mupirocin

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.

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

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*[55].

2.2. Tetracycline

Tetracycline inhibits binding of RNA to ribosomes[56] mainly by influencing binding to the A-site although some reports implicate binding of Ac-Phe-tRNA to the P-site[57,58]. A strong binding site exists on the 30S subunit and a lot of weaker sites on the 30S and 50S subunits[59–61]. Tetracycline may incorporate mainly into ribosomal proteins[62,63] since, in the absence of ribonucleoprotein, 16S RNA and the proteins S3, S7, S8, S14 and S19 show high affinity for tetracycline[64] particularly S7[60]. In addition, using a photoreactive benzophenone derivative of tRNA [3-(4-benzoylphenyl)propionyl-phenylalanine transfer RNA (BP-Phe-tRNA)], the photoreaction of 23S RNA was completely inhibited by tetracycline and tetracycline itself interacted efficiently with the loop V region of 23S RNA[65,66]. 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[67].

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[15,68]. 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 tranferase loop has a complex tertiary structure probably containing the adjacent hairpin loop[69,70]. 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 lincosamide 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[71].

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[72,73] to be on the 50S ribosomal subunit where chloramphenicol interacts with the central loop of 23S rRNA domain V to inhibit peptidyl transferase activity[68,71,72]. Details of the binding to the 50S subunits in *Deinococcus radiodurans* and *Haloarcula marismortui* have been revealed by X-ray studies[74,75]. Mutations in RNA can affect chloramphenicol binding[76,77].

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[78] 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 (ARE) 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[78].

Certain mRNAs also use allosteric control to mediate regulatory responses[79–84]. 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[85]. 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[86–88].

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[89]; (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 alboniger* which causes premature chain termination during translation in the ribosome. Part of the puromycin molecule resembles the 3′ end of the aminoacylated tRNA[90] such that it enters the A-site and transfers to the growing chain leading to an immature puromycylated chain and its premature release[91]. 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[92,93].

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*[94].

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 aaRSs including their crystal structures and active sites. Clearly they represent promising targets for novel antibiotics[95].

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 system[96,97]. Mupirocin is a secondary metabolite produced during the late stationary phase[98] 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 orthologs[99]. There are two genes (ileRS1 and ileRS2) in *P. fluorescens* which display remarkable differences[100]. 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 attack[100]. 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 infections[101]. Structural and energetic aspects of the binding of aristololactam-β-D-glucoside and daunomycin to tRNA(phe) have been investigated using various biophysical techniques[102].

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 spectroscopy[27]; a technique based on ESI-MS (IBIS Therapeutics) can screen tens of thousands of compounds in one day[29,103] but its high cost limits its wider application; SPR is a sensitive and convenient technique to monitor the binding to RNA of up to 100 small molecules per day.

There are also methods to establish the binding situation of small molecules to RNA in the absence of RNA-binding proteins. For example, the scintillation proximity assay (SPA) is a means of screening inhibitors of RNA–protein interaction[12]. The technique uses labeled complementary oligonucleotides to monitor the changes in the equilibrium between the folded and unfolded state of an RNA stem-loop structure produced by the binding of a small molecule[104]. In cells, the interaction between RNA and proteins can be measured using reporter gene fusion[105]. Moreover, a novel method to identify new compounds that disrupt the function of ribosomes in *Mycobacterium tuberculosis*[106] has identified two compounds (T766 and T054) with potent specific activity 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.

Acknowledgments

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