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Chemical and Functional Diversity of Small Molecule Ligands for RNA

Abstract: *Functional RNAs such as ribosomal RNA and structured domains of mRNA are targets for small molecule ligands that can act as modulators of the RNA biological activity. Natural ligands for RNA display a bewildering structural and chemical complexity that has yet to be matched by synthetic RNA binders. Comparison of natural and artificial ligands for RNA may help to direct future approaches to design and synthesize potent novel scaffolds for specific recognition of RNA targets.* © 2003 Wiley Periodicals, Inc. Biopolymers 70: 4–18, 2003

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INTRODUCTION

The continuing discovery of noncoding and structurally well-defined RNAs that participate in functional roles, rather than as coding mRNA sequences, has firmly established the acknowledgment of RNA as a key player in many cellular processes.[1–7] These functions are accomplished by ribonucleoprotein complexes in which RNAs interact permanently or transiently with proteins. Small molecule ligands have evolved in nature, predominantly as antibiotic secondary metabolites of fungal and bacterial origin, which can modulate or inhibit cellular processes by interacting specifically with the RNA components of ribonucleoprotein complexes.[8–11] The paradigm of RNA–small molecule interaction is provided by the bacterial ribosome that is the target for many natural antibiotics that bind to ribosomal RNA (rRNA) and thereby interfere with bacterial protein synthesis.[8,12–15] Artificial RNA aptamers for a chemically diverse variety of small molecules have been obtained by in vitro evolution, demonstrating the versatility of RNA in ligand recognition[16] and suggesting that RNA–small molecule interactions might play a role in biological processes other than translation. Recently, direct binding of small molecule coenzymes to the structured 5′ untranslated regions of some mRNAs has been discovered,[17–19] confirming earlier hypotheses of such interactions.[20]

The realization that rRNA is the primary target for many ribosome-directed antibiotics,[8,12–14] in combination with the recent determination of three-dimensional structures of the bacterial ribosome,[21] along with earlier findings that some antibiotics specifically recognize viral RNA structures,[22,23] have spurred the interest in RNA as a drug target.[10,24] Albeit the ratio-

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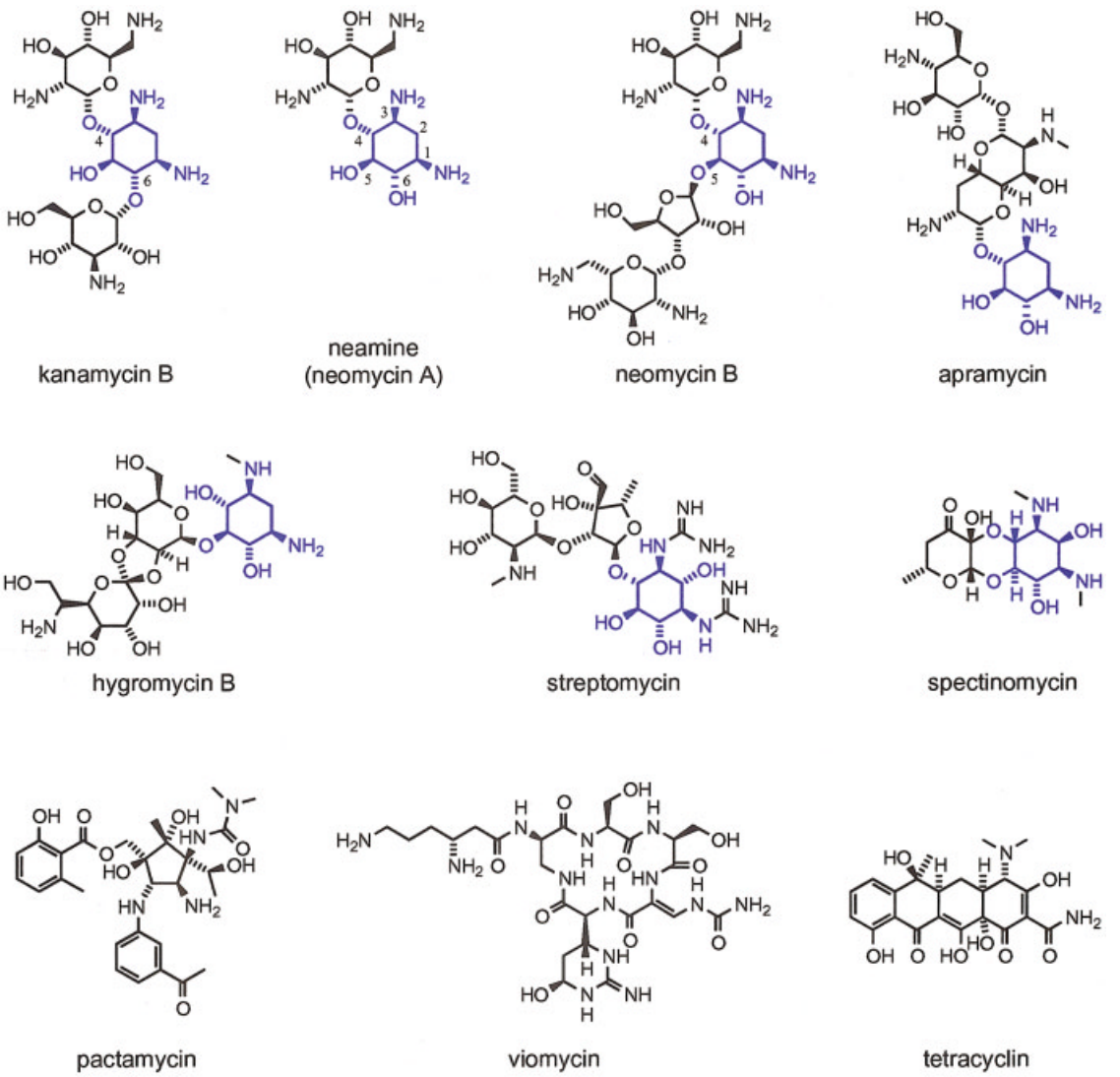

FIGURE 1 Ligands of 16S ribosomal RNA. The conserved 2-DOS and streptamine core scaffolds of the aminoglycoside antibiotics are marked in blue.

nal discovery of novel small molecule ligands for RNA is still in its infancy, compared to the well-tried routes to protein enzyme-directed drug design, efforts are increasingly directed at the design, synthesis, and screening for powerful modulators of cellular functions acting on RNA targets.[25–30] From these studies it has become clear that the particular chemical and structural characteristics of RNA require fresh perspectives on drug discovery, deviating from the established wisdom of ligand design for proteins. The primary source of knowledge on molecular features for the recognition of RNA targets are natural antibiotics known to interact with the bacterial ribsome. Here, I want to give a brief overview on the chemical diversity of natural small molecule ligands for RNA, and draw a comparison to the chemical classes of synthetic compounds that have been reported in the literature.

AMINOGLYCOSIDE LIGANDS OF THE SMALL RIBOSOMAL SUBUNIT (30S)

The 16S rRNA in the small ribosomal subunit is the target for a variety of aminoglycoside antibiotics (Figure 1).[8,12—14] The natural aminoglycosides share in common the 2-deoxystreptamine (2-DOS) or streptamine core carrying amino-sugar substitutents at different positions. The 4,6-disubstituted 2-DOS derivatives of the kanamycin class and the 4,5-disubstituted 2-DOS compounds of the neomycin series are the best studied types of aminoglycoside antibiotics.

They bind to the A-site internal loop within the deep groove of helix H44 of 16S rRNA[31] and interfere with two conformationally flexible adenine residues (A1492, A1493),[32] which are involved in the selection of cognate aminoacyl–tRNA during translation, thereby increasing the misincorporation of near-cognate amino acids. Three-dimensional structures of aminoglycosides from both the 4,6- and 4,5-disubstituted 2-DOS series bound to the A-site RNA reveal a conserved pattern of molecular recognition that involves exclusively RNA.[31–35] The 2-DOS ring faces edge-on base pairs within the deep groove of the A-site RNA helix. The glucosamine substituent at the 4-position lies within the base-stacking plane, forming a base-pair-like interaction with an adenine (A1408).[33] These molecular contacts are sufficient to determine A-site specificity, attested by the antibiotic potency of neamine,[36] a natural 2-DOS compound with a single amino-glucosamine substitution at the 4 position. Additional RNA contacts of sugars at the 6 or 5-position in the disubstituted 2-DOS derivatives contribute to binding affinity but are less important for achieving target specificity. Amino groups of the aminoglycosides, most of which are protonated and positively charged at physiological pH,[37] displace magnesium ions by docking to metal ion binding sites of the uncomplexed rRNA, as revealed in the crystal structure of the 30S subunit in complex with mRNA and cognate tRNA.[32]

The A-site-binding aminoglycoside apramycin is related to neamine as a 4-substituted 2-DOS compound, however carrying an unusual bicyclic sugar modification that adopts a *trans*-decalin-like conformation. Upon binding to its RNA target, apramycin interferes with the flexible adenines A1492 and A1492 as well, perhaps by locking one of the bases within the RNA helix.[38]

The 5-substituted 2-DOS derivative hygromycin B binds to the deep groove of helix H44 in 16S rRNA, just above the binding site of the kanamycin and neomycin class aminoglycosides, where it interacts exclusively with RNA bases.[39] It has been suggested that hygromycin B sequesters A-site-bound tRNA by restricting a conformational change in H44 that is required for ribosome translocation.[39] Compared to other aminoglycoside antibiotics, hygromycin B is more rigid due to its spiro-acetal functionality that connects the two sugar substituents of 2-DOS.

The most rigid of the aminoglycosides that are discussed here is the tricyclic spectinomycin which is formally derived from a 4,5-disubstituted streptamine core by combination of two vicinal acetal linkages to the same spectinose sugar. The *cis* arrangement and axial conformation of the two acetal linkages forces the spectinose moiety out of plane and in a distorted conformation. Unlike the compounds described above, spectinomycin binds in the shallow groove, at one end of helix H34 in the 3′ major domain of 16S rRNA.[31] It interacts exclusively with RNA moieties and inhibits elongation-factor-G-catalyzed translocation of the peptidyl–tRNA from the A site to the P site of the ribosome.[31] Similar to hygromycin B, spectinomycin may act as a rigid molecular brace that prevents a conformational change in an RNA helix (H34).

The streptamine derivative streptomycin carries a single glycosidic linkage at the 4-position and two guanidinium substituents that enhance the hydrogen-bond donor capacity of the streptamine core. Streptomycin binds tightly to the small ribosomal subunit, forming contacts to rRNA helices H27, H18, H44, and the S12 protein.[31] The close proximity of phosphate groups to the furanose in the rRNA-bound drug suggests that the aldheyde group of streptomycin is perhaps hydrated, increasing the number of hydrogen bonds to the RNA backbone, which is in line with the retained antibacterial potency of dihydrostreptomycin. With its multiple interactions at the intersection of several RNA domains and one protein, streptomycin is an example par excellence of a noncovalent molecular cross-linker that incapacitates the works of the ribosome machinery. As a consequence, streptomycin stabilizes the *ram* state (ribosomal ambiguity) of the ribosome and intereferes with both the initial tRNA selection and proofreading.[8]

The aminoglycosides that have been discussed so far provide rigid molecular scaffolds for the presentation of basic groups and hydrogen-bond donor moieties that participate in an intimate network of interactions with the ribosomal RNA targets. Molecular recognition of the aminoglycosides via polar hydrogen bonds is finely tuned by modulation of the basicity of amino groups,[40,41] depending on monomethylation (as in apramycin, hygromycin B, spectinomycin, and the gentamicins), the presence of vicinal hydroxyl groups, or additional guanidinium substituents (as in streptomycin). Several aminoglycosides, notably neomycin B with its six amino groups, have been shown to interact with affinities in the micromolar range with nonribosomal RNA targets[42,43] such as self-splicing group I introns,[44] hammerhead[45,46] and hepatitis delta virus (HDV)[47] ribozymes, bacterial RNase P,[48] tRNA,[49–51] and viral regulatory RNA domains such as HIV-1 trans-activating response element (TAR),[23] the Rev-response element (RRE),[22] and the packaging region (ψ-RNA)[52] and dimerization initiation site RNA.[53] The promiscuity of natural aminoglycoside antibiotics for recognition of a variety

of RNA targets has been attributed to structural electrostatic complementarity between the positive charges on the aminoglycoside scaffolds and the negative charge distribution in RNA folds.[40,54]

NONAMINOGLYCOSIDE LIGANDS OF THE SMALL RIBOSOMAL SUBUNIT (30S)

Pactamycin (Figure 1) is a highly substituted cyclopentane derivative that interacts with hairpin loops H23b and H24b of 16S rRNA, close to the tRNA-binding cleft.[39] It has been suggested that pactamycin may act as a dinucleotide mimic that partially displaces mRNA from the ribosomal E site.[39] Sterical crowding of substituents at the cyclopentane core forces the two aromatic moieties in pactamycin to stack against each other, giving rise to a rigid dinucleotide-like molecule. Indeed, pactamycin binds to the rRNA by consecutive stacking of the aromatic rings against a guanine base and additional interactions of the polar-substituted cyclopentane core that mimics to some extent the RNA sugar–phosphate backbone.[39]

Tetracylines are an important class of antibiotics that are still frequently used in therapy of bacterial infections. The parent compound (Figure 1) is an octahydronaphtacene derivative with an overall L-shape, distorted by a kink that forces the outer *cis*-linked cyclohexenone ring out of plane. The two long edges of tetracycline display a remarkable difference in the distribution of polar groups, giving rise to a distinct partition between preference for hydrogen bonding on one face and for hydrophobic interactions on the other. Several tetracycline binding sites have been identified in the crystal structures of the small ribosomal subunit,[39,55] perhaps attesting the potential of overall flat molecules for generic recognition of clefts in RNA folds and RNA–protein complexes. At the primary binding site of tetracycline, which has been attributed to its biological function of interference with aminoacyl–tRNA binding to the A site, the drug interacts with 16S rRNA in the shallow groove of helix H34 including contacts to helix H31.[39,55] A magnesium ion chelated between the keto and enol groups of the drug bridges between tetracycline and the rRNA,[39,55] similar to a magnesium-mediated interaction in the *tet*-repressor–DNA structure.[56] Chelating of divalent cations is required for tetracycline binding to both the ribosome and the *tet*-repressor.

Viomycin is a cyclic hexapeptide antibiotic of the tuberactinomycin family that contains several unusual amino acids, among them diaminopropionic acid and a cyclized arginine (Figure 1). An intramolecular hydrogen bond between the arginine amide proton and a serine carbonyl group enhances the rigidity of the 16-membered peptide ring which adopts a β-turn conformation both in solution and in the crystal.[57] Upon binding to the ribosome, the antibiotic stabilizes subunit cohesion and inhibits translocation by sequestration of peptidyl–tRNA in the A site.[57] Chemical probing has mapped the viomycin binding site to the ribosomal subunit interface, in proximity to the A site, and contacting the central pseudoknot of 16S rRNA.[57] Similar to the aminoglycoside antibiotics, viomycin binds with affinities in the micromolar range to other nonribosomal RNA targets. The binding sites of the peptide in self-splicing group I introns,[58] HDV ribozymes,[47] and an artificial viomycin-binding aptamer[59] contain RNA pseudoknots, suggesting that viomycin may recognize pseudoknot structures.[59] Viomycin also binds to the hepatitis C virus (HCV) internal ribosome entry site (IRES) RNA with micromolar affinity, yet not to a pseudoknot motif.[60]

LIGANDS OF THE LARGE RIBOSOMAL SUBUNIT (50S)

Most antibiotics that are known to bind to the large ribosomal subunit (Figure 2) interact at or in immediate proximity of the peptidyl transferase center, a multijunctional loop in domain V of 23S rRNA. Thus, the binding sites of these compounds are in some cases overlapping, and chemical probing and resistance mutation data do not allow to clearly map the precise interaction target for each drug. Binding sites are unambiguously known for compounds that have been used in x-ray crystallographic structure studies of ribosome–antibiotic complexes,[61,62] including several macrolides, clindamycin, and the 3′-aminoacyl-–tRNA mimetics chloramphenicol, sparsomycin, and puromycin (Figure 2).

The macrolides are macrocylic lactone antibiotics that are widely used as drugs in antibacterial therapy.[63] Most macrolide antibiotics belong to one of the three chemical classes shown in Figure 2, comprising 14-, 15-, and 16-membered natural and synthetically modified lactones and azalides. The 15-membered azalides, such as azithromycin, are obtained semisynthetically from 14-membered lactones by conversion of the carbonyl group at the 9 position into an oxime and subsequent Beckmann rearrangement to the expanded aza-lactone (azalide). The macrolides bind to 23S rRNA at the entrance to the polypeptide exit tunnel, immediately adjacent to the peptidyl transferase center, and thereby physically block the egress

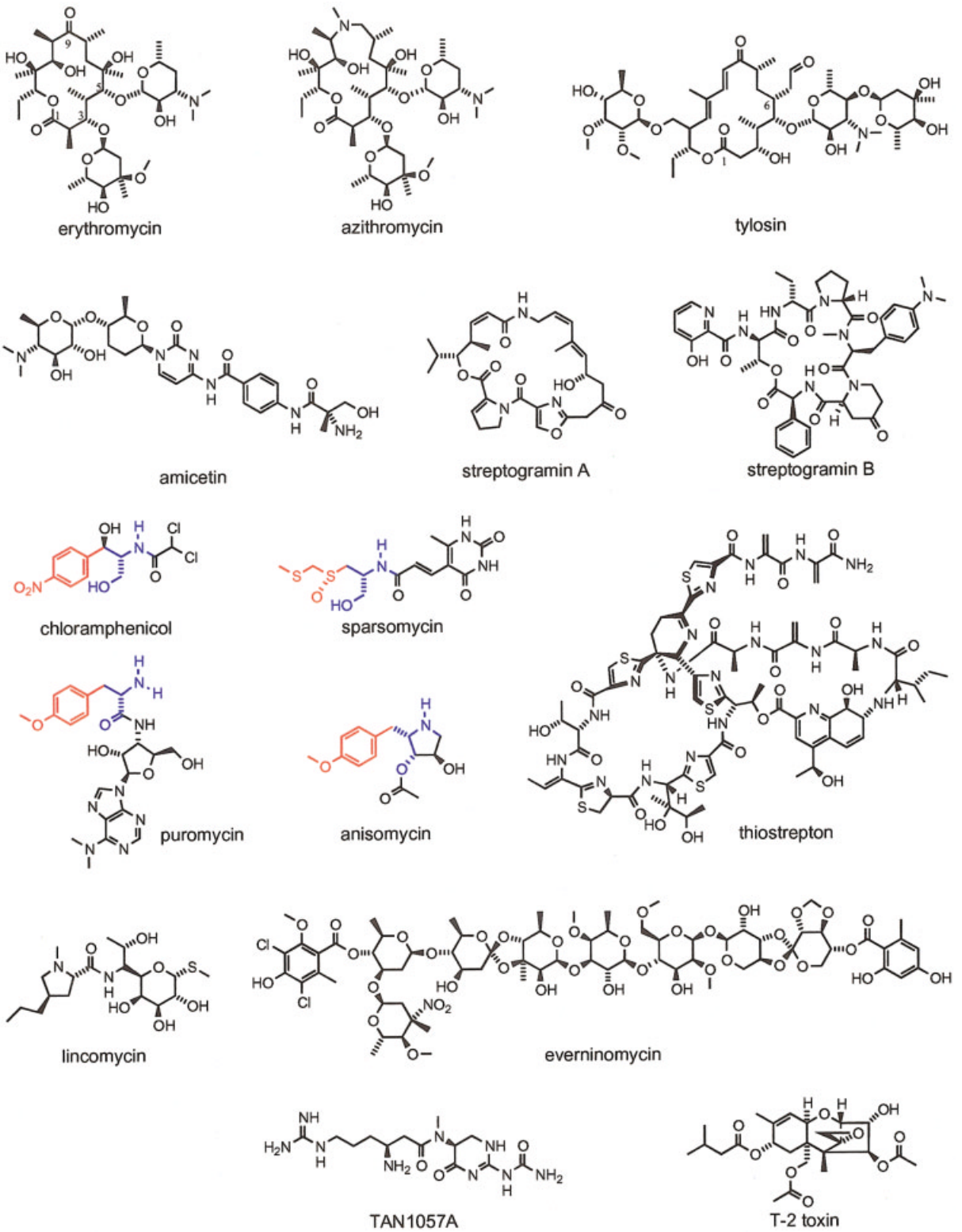

FIGURE 2 Ligands of 23S ribosomal RNA. In the antibiotics chloramphenicol, sparsomycin, puromycin, and anisomycin, which function as structural analogs of the 3′-terminus of aminoacyl–tRNA, putatively isostructural motifs are marked by coloring (hydrophobic domain in red; amino-acceptor moiety in blue).

of nascent protein.[61,62] Crystal structure and NMR analyses of macrolides free and in complex with the large ribosomal subunit reveal that the macrocyles retain a single low-energy conformation both free in solution and when bound to the ribosome.[62] The lactone ring adapts a folded-out conformation that projects most polar groups pointing away from the center and towards one face of the molecule, whereas the opposite side of the macrocycle is quite hydrophobic. The apolar face of the macrolide ring is oriented toward the hydrophobic wall of the peptide exit tunnel, interacting with residues of the L4 and L22 proteins, and exposing the hydrophilic side of the drugs toward to solution.[62] Crystal structure analyses of 50S ribosomal subunit-macrolide complexes unexpectedly revealed a covalent bond between the aldehyde group at the 6-position of the 16-membered macrolides and an adenine N6 atom.[62] The exocyclic amino group of adenine may react reversibly with the macrolide carbonyl functionality to form a hemiaminal, which is stabilized by the adjacent aromatic system of the nucleobase. The sugar substituents of the macrocylic core scaffolds play a major role for the binding affinity of the antibiotics, attested by their significant contributions of one-half to two-thirds of the intermolecular contact surface in the ribosome–drug complexes.[62] The saccharide moieties at the 5-position of the lactone core, deoxy-mycaminose (desosamine) in both erythromycin and azithromycin, and a cladinose-substituted mycaminose in tylosin, extend from the macrolide binding site towards the all-RNA environment of the peptidyl transferase center where they interact exclusively with nucleotides of 23S rRNA.[61,62] In contrast to the aminoglycoside antibiotics that have been discussed above, the saccharide components of the macrolides contain only a single amino functionality, the dimethylamino group of the mycaminose sugar, which is thought to be protonated at physiological pH.[61] As a consequence, the macrolides have an overall lower basicity, resulting in their favorable pharmacokinetic profiles and oral bioavailability. The lower density of basic groups in the macrolides reflects their binding mode as ligands bridging between ribosomal RNA and proteins, which exploit the recognition of structural features at the interface of ribonucleoprotein complexes.

The location of the mycaminose sugar in the structure of the 50S–macrolide complexes overlaps with the position of the thiosugar of lincosamide antibiotics bound to the large subunit. The lincosamides, such as lincomycin (Figure 2) and clindamycin, bind to the peptidyl transferase loop in 23S rRNA and contact exclusively RNA residues.[61] Similar to chloramphenicol and the aminoglycosides, lincosamides displace a magnesium ion from the native RNA upon binding and interfere both with the positioning of the aminoacyl group at the A-site-bound tRNA and the peptidyl group at the P-site tRNA.[61]

The proline residue of lincosamides partially overlaps the position of the chloramphenicol aromatic ring in 50S complexes. Lincosamides, chloramphenicol, sparsomycin, puromycin, and anisomycin (Figure 2) bind competitively to the large ribosomal subunit.[64–67] The four latter antibiotics belong to different chemical classes that share roughly coinciding binding sites on the peptidyl transferase center of 23S rRNA but interfere with translation by distinct mechanisms. Sparsomycin, puromycin, and anisomycin are universal inhibitors of peptide bond formation in eubacteria, archaea, and eucarya, whereas chloramphenicol acts predominantly on bacterial translation. The four antibiotics are partially isostructural (Figure 2), comprising an apolar moiety connected by a two-carbon linker to an amino or amide nitrogen atom. Binding of the lipophilic part into a hydrophobic pocket orients the nitrogen functionality such that it competes with the correct positioning of the aminoacyl–tRNA acceptor. Chloramphenicol blocks the peptidyl transferase activity by sterical interference with the aminoacyl moiety in the A site and thereby prevents the formation of the transition state during peptide bond formation.[61] The drug interaction is exclusively with nucleotides of the rRNA, involving the displacement of an RNA-bound magnesium ion, but also the creation of a novel magnesium ion binding site between the chloramphenicol primary hydroxyl group and the RNA.[61] Puromycin acts both as structural and functional analog of aminoacyl–tRNA since its primary amino group allows it to function as an amino acid acceptor substrate and terminator of peptide elongation.[68] The heterocyclic base substituent at the furanose sugar is likely to interact with rRNA by stacking, perhaps isostructural to the adenine of the tRNA 3′-terminus, but not by base pairing.[68] The methyl-uracil residue of sparsomycin is involved in a similar stacking interaction.[69,70] Sparsomycin binds to ribosomes only in the presence of peptidyl–tRNA in the P site, which is in turn stabilized by the bound antibiotic and prevented from proceeding into the peptidyl-transfer step.[64] Interaction of anisomycin with the 23S rRNA inhibits peptide bond formation by sterical interference with the aminoacyl–tRNA 3′ acceptor.[67] In eukarya, anisomycin binding to the 28S rRNA additionally triggers a ribotoxic stress response that induces stress-activated protein kinases (SAPKs), also known as c-Jun N-terminal kinases (JNKs).[71] Whereas the molecular mechanism of this trigger is unknown, it has been

suggested that induction of a conformational change in the rRNA in response to anisomycin binding elicits the signal for activation of SAPK/JNK.[71] Trichothecenes, such as T-2 toxin (Figure 2), which interact with the peptidyltransferase center of rRNA,[65] albeit their precise target site is less well studied, can also inhibit protein synthesis and activate JNK by the ensuing ribotoxic stress.[72]

Similar to some of the antibiotics that have been discussed above, amicetin (Figure 2) is a universal inhibitor of peptide bond formation.[73,74] The drug binds in close proximity of the peptidyl transferase center of 23S rRNA, at a conserved motif that overlaps with the chloramphenicol binding site but not with the macrolide target region.[73] Amicetin is a cytosine derivative carrying both a peptide substitutent at the exocylic amino group and sugar moieties linked to the base.

Recently, the dipeptide antibiotic TAN1057 (Figure 2) has been described[75] that blocks translation in bacteria and eukarya by inhibiting the peptidyl transferase activity.[76] While the interaction site of the antibiotic has not been mapped, competition binding experiments indicate that the TAN1057 target does not overlap with the binding sites of chloramphenicol or the macrolides.[76] TAN1057 has not been considered an attractive drug candidate because of its toxicity for eukarya and since the dipeptide requires an active transport mechanism for uptake into cells, rendering it highly susceptible to development of resistance in components of the transport machinery.[76]

Streptogramin A and B (Figure 2) are cylic peptide–lacton compounds that otherwise share little common chemical similarity. Interest has been revived recently in these antibiotics, which are emerging as important drugs for antibacterial therapy.[77] The interaction site of the antibiotics has been mapped to the peptidyl transferase loop of 23S rRNA.[78] Mixtures of the streptogramins inhibit peptide elongation synergistically, perhaps by streptogramin B blocking the passage of the nascent peptide in a fashion similar to the macrolides.[78] The action of streptogramin A on the ribosome is less well characterized. It has been suggested that it may stimulate the binding of the B component to the peptidyl transferase center by stabilizing an RNA conformation with increased affinity for the antibiotic.[78]

The oligosaccharide antibiotic everninomycin (Figure 2) is the best characterized compound of the orthosomycins, which inhibit bacterial translation by preventing the interaction of IF2 with the large ribosomal subunit.[79] The binding site of everninomycin has been mapped to hairpin loops H91 and H89, which are extending from the multijunctional peptidyl transferase center.[79,80] It has been proposed that the aromatic moieties in the antibiotic may interact with RNA bases by stacking or intercalation.[79] Resistance mutations indicate that residues of the L16 protein might form contacts to everninomycin as well.[79] Among the antibiotics discussed here, everninomycin along with the cyclic peptide thiostrepton, are the largest molecular scaffolds that target rRNA, having a molecular weight in excess of 1500D.

Thiostrepton (Figure 2) is a thiazole–peptide antibiotic that inhibits the ribosomal GTPase center, thereby shutting down all GTP-dependent reactions during translation.[81–83] The complex bicyclic structure of the peptide is organized around a propeller-shaped central scaffold, which consists of an unsaturated piperidine moiety directly linked to three thiazole rings. The piperidine appears as a cross-link, dissecting the cyclic peptide into one half that comprises several thiazole groups and the other half that contains a substituted dihydro-quinaldic acid building block. A linear peptide of two dehydroalanine units extends from one of the thiazoles linked to the piperidine core. Whereas the water-insolubility of thiostrepton has so far prevented the co-crystallization with the ribosomal target, biochemical methods have been extensively used to study ribosome binding and activity of the antibiotic.[81–87] Thiostrepton interacts with the 50S subunit at the GTPase-associated domain of 23S rRNA, which is the independently folding binding site of the L11 protein. Biochemical data along with molecular modeling studies suggest that the cyclic peptide moiety binds at the interface between RNA and L11 protein, and that the linear extension of thiostrepton interacts with RNA in a narrow deep pocket formed between two hairpin loops of the L11-binding domain.[84,87] This pocket is lined by phosphate groups, which create an electronegative environment suitable for the binding of cations in the absence of thiostrepton.[88–90] Interestingly, it has been shown that both magnesium ions and thiostrepton stabilize the same native three-dimensional fold of the GTPase-associated domain RNA.[84] The biological function of thiostrepton as inhibitor of the ribosomal GTPase has been attributed to its stabilizing interaction with the GTPase domain RNA–L11 protein complex, which might prevent a conformational change required for GTPase action.[84] It is not clear, however, whether the antibiotic blocks primarily a conformational change in the RNA, or in the L11 protein, or both.

NATURAL LIGANDS OF 5′-UNTRANSLATED REGIONS IN mRNA

For many of the above discussed antibiotics, interaction with the ribosomal RNA was confirmed long

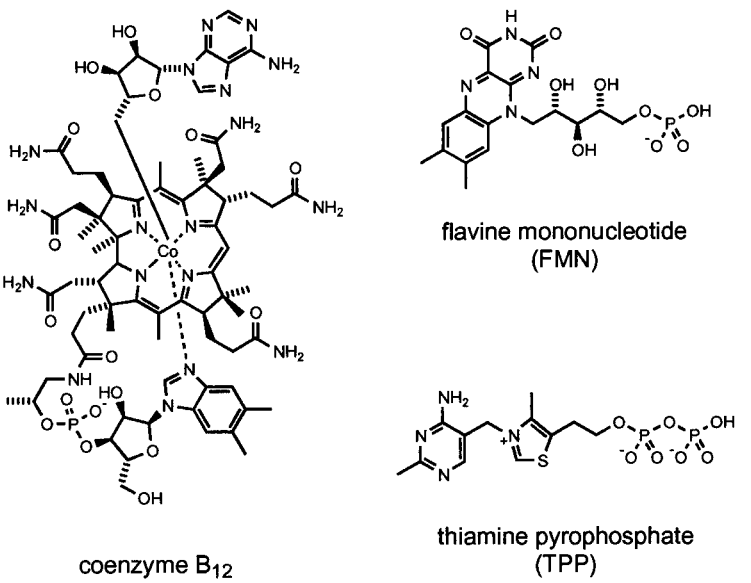

FIGURE 3 Ligands of 5′-untranslated regions in mRNAs involved in bacterial coenzyme biosynthesis.

after their inhibitory effect on translation had been uncovered. After the seminal discovery that aminoglycosides recognize directly the 16S RNA of the small ribosomal subunit,[12–14] the paradigm of RNA as a target for small molecule ligands has been firmly established.[10,15,24,28,29] In vitro selection of RNA aptamers for a chemically diverse variety of ligands has since demonstrated the versatility of RNA molecular recognition.[16] These findings, along with the postulated key role of RNA as an enzymatically active species during a prebiotic RNA world,[91–93] have fostered the expectation that small molecule–RNA interactions may be more widespread in the regulation of biological RNA functions. It has been suggested that in the prebiotic RNA world small molecules might have acted as modulators of functional RNAs.[11,94] Whereas the above discussed antibiotics represent secondary metabolites, which are synthesized and excreted by fungi or bacteria for defense against other organisms, small molecule–RNA interactions may also be conceived playing a role in innate regulatory processes.

Artifical ligand-regulatable operons have been constructed by inserting small molecule aptamers in the untranslated region (UTR) between 5′ cap and start codon of mRNAs.[95] Recently, natural systems of ligand-controlled mRNA translation have been discovered in bacteria.[17–19] It has been found that the translation of mRNAs that code for enzymes involved in the biosynthesis and metabolism of some vitamins is regulated by binding of vitamin cofactors to structured domains within the 5′-UTR. Such direct regulatory interactions between mRNAs and cofactors without the mediation of proteins were discovered for thiamine (vitamin B_1),[18,19,96] riboflavin (B_2),[18,19] and coenzyme B_{12}[17,97,98] (Figure 3). Thiamine pyrophosphate (TPP), the active form of coenzyme B_1, binds with nanomolar affinity to the conserved *thi*-box domain in the untranslated leader region of *thiC* and *thiM* mRNAs,[18,19] which encode thiamine biosynthetic proteins in *Escherichia coli* and *Bacillus subtilis*. Cofactor binding to the *thi*-box RNA induces a secondary structure rearrangement that sequesters the Shine–Dalgarno sequence within a helix, thereby precluding it from ribosome access.[18,19] The term "riboswitch" has been coined for the conformational adaptation of the *thi*-box RNA upon binding of TPP, which resembles the adaptive structure formation processes frequently observed in aptamer–ligand interactions.[18] Structure–affinity relationships of thiamine derivatives suggest that the exocyclic amino group of the pyrimidine moiety and the phosphate groups of TPP contribute to the binding to RNA.[18] Whereas the binding site of TPP within the *thi*-box RNA is not yet known with precision, it is conceivable that the thiamine pyrimidine group is involved in pairing or stacking interactions with RNA bases.

Similar regulatory ligand–mRNA interactions have been discovered for flavin mononucleotide (FMN, Figure 3),[18,19] which regulates expression of genes involved in riboflavin biosynthesis in *B. subtilis*, and for coenzyme B_{12} (cobalamin, Figure 3),[17] which represses the cobalamin biosynthetic *cob* operon in *Salmonella typhimurium*. The binding sites for FMN

and cobalamin have been mapped to the 5′-UTR of the mRNAs,[17–19] but their molecular architecture is unknown. However, isoalloxazine derivatives, such as FMN, have been shown earlier to recognize structural signatures within the shallow groove at consecutively stacked GoU base pairs in A-form RNA helices.[99] Coenzyme B_{12} has also been found to inhibit translation of hepatitis C viral (HCV) mRNA,[100,101] which is initiated cap-independently from an internal ribosome entry site (IRES). Biochemical and footprinting data suggest that coenzyme B_{12} can bind directly to the IRES RNA, perhaps by recognizing a pseudoknot structure.[101] It is not known, however, if the HCV IRES and the 5′-UTR of *cob* mRNA share structural similarities that are recognized by the coenzyme.

Interestingly, the three cofactors for which direct binding to natural RNAs has been demonstrated, contain negatively charged phosphate groups. In the case of the thiamine interaction with *thi*-box mRNA, structure–affinity relationships of thiamine derivatives demonstrate the importance of the phosphate groups for ligand–RNA association.[18] The binding affinity for thiamine–phosphate is reduced to the micromolar range as compared to TPP, and thiamine itself, which does not carry a phosphate, shows a more than 1000-fold lower affinity for the *thi*-box RNA. The dependence of ligand affinity on the presence of phosphate groups might be due to cation-mediated specific interactions between TPP and its target site in the mRNA, similar to the participation of magnesium ions in rRNA binding of tetracylines and chloramphenicol.

SYNTHETIC LIGANDS FOR RNA

The demonstration that rRNA is the molecular target for ribosome-directed aminoglycoside antibiotics,[12–14] followed by the discovery of aminoglycoside binding to regulatory elements RRE[22] and TAR[23] in HIV mRNA, have motivated systematic approaches to design, synthesize, and screen small molecules as ligands for functional RNAs. The concept of exploiting RNA as a drug target has been subject to a number of previous reviews, which provide also outlines of current approaches to discover RNA-specific small molecule ligands.[10,11,15,24–30] Here, I want to concentrate on the chemical classification of synthetic ligands for RNA that have emerged from these efforts (Figure 4), along with their comparison to natural RNA binders.

Due to their ability to recognize different RNA structures, aminoglycoside antibiotics provide obvious starting points and templates for the synthesis of novel RNA-directed ligands. Numerous attempts to obtain new ligands for the ribosomal A site have focused on simplifying the highly functionalized aminoglycosides by synthetic modification of core scaffolds that are conserved among the natural compounds. The goal of these approaches was to develop compounds of lower structural complexity, which first, are amenable to straightforward medicinal chemistry exploration, second, have more favorable pharmacological profiles than the aminoglycosides, and third, are less sensitive to bacterial resistance mechanisms. Comprehensive overviews on modified natural aminoglycosides and synthetic analogues which were derived from amino-glucosamine (**1**, Figure 4), 2-DOS (**2**), paromamine, and neamine (**3**) have been published previously, covering the literature until 2002.[29,30,102–104] In summary, none of the simplified synthetic aminoglycoside analogues matched in RNA binding affinity and antibacterial potency the more complex natural aminoglycosides such as neomycin B (Figure 1). Moderately potent compounds were found more frequently among analogues that retained the two-ring aminoglycoside system of neamine or paromamine, whereas the most simplified derivatives of glucosamine or 2-DOS were rarely active. Systematic studies on the role of the hydroxyl and amino groups in modified natural aminoglycosides revealed a finely tuned interplay between these functionalities.[40,41] In particular, neighboring hydroxyl groups modulate the basicity of amino groups such that high RNA binding affinity is obtained without sacrificing target specificity.[40,41] While the increase of compound basicity leads to higher binding affinities—for example, in guanidino-aminoglycosides (**4**, Figure 4), specificity is lost simultaneously.[105] Even slight modifications, such as the substitution of the neamine 6′-amino group by other functionalities, can lead to drastic reduction of compound activity on the A-site target.[106] The abundance of functional groups in aminoglycoside antibiotics, thus, appears to be a result of their status as evolutionary highly optimized scaffolds for RNA recognition rather than a remnant of excess as a consequence of their biosynthetic origin.

In alternative approaches to novel RNA-targeted ligands based on aminoglycosides, not aimed at simplifying the molecules but at increasing affinity by combination of binding motifs, dimers[107,108] and conjugates such as acridine–neomycin[109] (**5**, Figure 4) have been synthesized. These compounds are aimed at exploiting additive effects for increased RNA affinity by combining target-specific motifs, such as an aminoglycoside, with a generic nucleic acid binding motif, such as the intercalator acridine. Although these conjugate compounds often display higher RNA

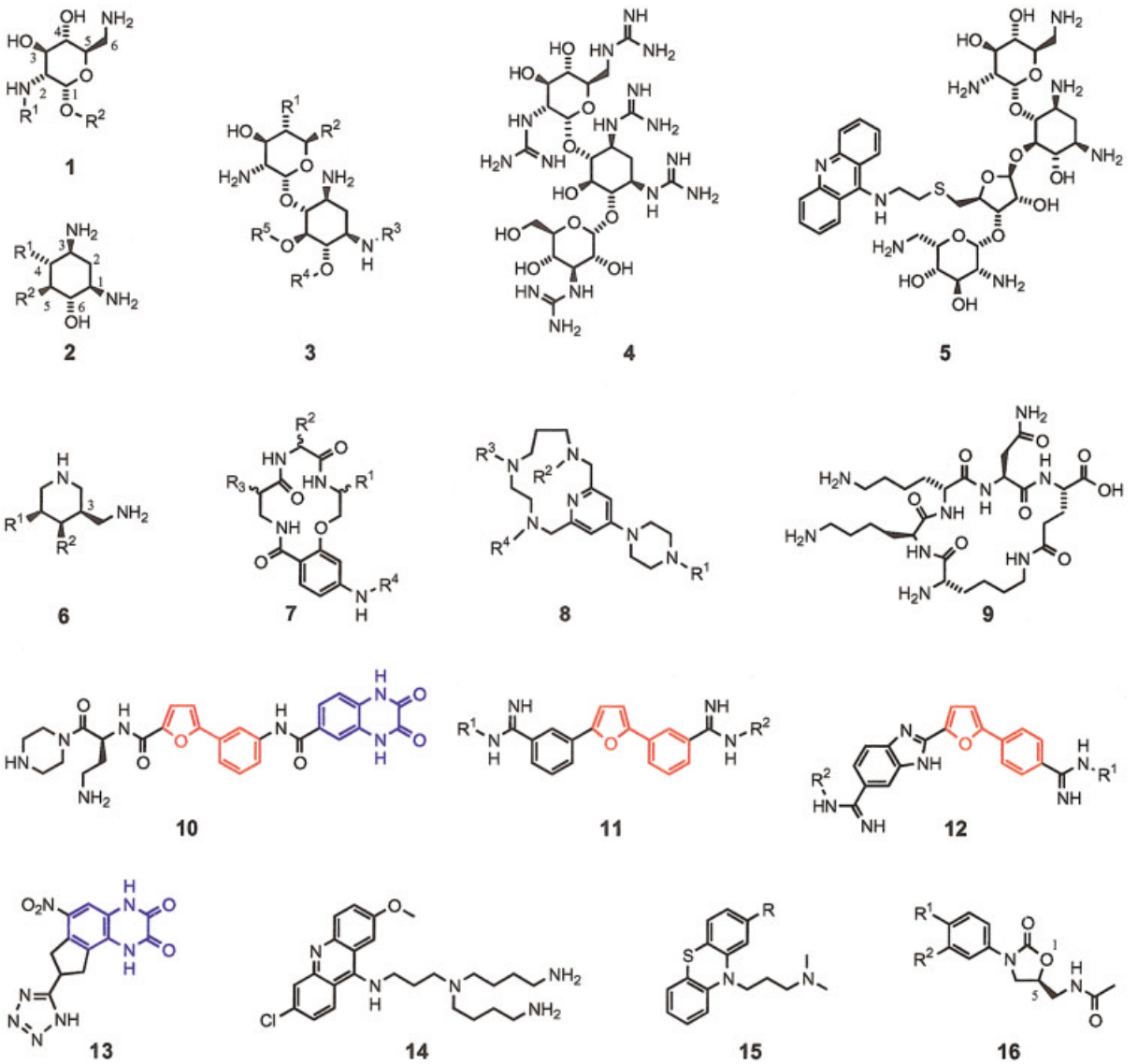

FIGURE 4 Synthetic ligands of ribosomal and viral RNA targets. **1**, 6-amino-glucosamine derivatives; **2**, 2-DOS derivatives; **3**, neamine (R^2=CH_2NH_2) and paromamine (R^2=CH_2OH) derivatives; **4**, guanidino-kanamycin A; **5**, acridine–neomycin conjugate; **6**, 3-aminomethyl–piperidine derivatives; **7**, 14-membered macrocyclic amides (potent inhibitors of bacterial translation have R^4= acyl- or sulfonyl-linked quinoxaline-2,3-dione); **8**, 13-membered cyclic polyamines; **9**, HIV-1 TAR-targeted cyclic peptide; **10**, quinoxaline–2,3-dione derivative targeted at the GTPase-associated domain in 23S rRNA; **11**, diphenylfuran derivatives; **12**, benzimidazolyl–phenylfuran derivatives; **13**, HIV-1 TAR-targeted quinoxaline–2,3-dione derivative; **14**, aminoacridine derivative; **15**, phenothiazine derivative (phenazine) **16**, oxazolidinones. Most potent oxazolidinones follow the shown formula, although some active compounds with an alternative substitution scheme at the oxazolidinone and aromatic rings have been described. The quinoxaline–2,3-dione core common to the compound classes **10**, **13**, and **7** (at the R^4 position) is marked in blue. The phenylfuran scaffold shared between the ligands **10**, **11**, and **12** is colored red.

affinity than the parental aminoglycosides, their importance is mainly as tools to study ligand–RNA interactions, since the increased molecular mass and chemical complexity limits their potential as lead structures for drug development.

The ultimate challenge in ligand design for RNA targets such as the bacterial A site are approaches that abandon the conventional aminoglycoside chemistry in favor of novel RNA-friendly scaffolds that are not compromised by undesirable pharmacological profiles

and bacterial resistance. Towards this goal, 3-aminomethyl-piperidine derivatives (**6**, Figure 4) have been synthesized that were designed, based on crystal structure data, to mimic the spatial arrangement of amino groups in 2-DOS.[110] The inclusion of one amino group into a heterocycle in the aminomethyl-piperidines gave rise to altered vectors for substituents (**6**, R^1 and R^2, Figure 4) pointing off the cyclic core in directions distinct from 2-DOS. The piperidine derivatives that bound tightest to the A-site RNA and displayed the highest potency as inhibitors of bacterial in vitro translation, however, were similar to aminoglycosides while carrying an amino-glucosamine substitutent at the R^1 or R^2 position.[110]

Similar to the aminoglycosides, macrocyclic antibiotics such as the macrolides and streptogramins have served as templates for the design of novel potentially antibacterial and antiviral compound series targeted at RNA. Peptide chemistry or alkylation of amino functionalities have been used most frequently to construct synthetic macrocycles, including the antibacterial 14-membered amides **7**,[111] and the 13-membered polyamines **8**,[112] which were investigated as HIV-1 Tat–TAR inhibitors. The 14-membered macrocycles **7** yielded antibacterial compounds that inhibited in vitro translation assays, albeit their molecular target has not been determined. Interestingly, all translation inhibitors of the 14-membered amide series **7** had a quinoxaline-2,3-dione substituent at the R^4 position,[111] which was identified as a structural element required for RNA binding in synthetic ligands (**10**, Figure 4) of the GTPase-associated domain in 23S rRNA[113] and in an inhibitor (**13**, Figure 4) of the HIV-1 Tat-TAR interaction.[114] The potential to participate both in base-pairing and -stacking interactions might render the quinoxaline-2,3-dione moiety a privileged building block of RNA-targeted ligands. The tricyclic quinoxaline-2,3-dione derivative **13**, which was discovered in a screening program for Tat–TAR inhibitors,[114] is among potent RNA binders with the lowest molecular mass known so far. The compound disrupted the Tat peptide–RNA interaction at low micromolar concentrations, comparable to the potency of neomycin B.[114]

In the synthetic macrocycles, such as **7** and **8**, as well as the cyclic peptide **9**, which was described as an inhibitor of the HIV-1 Tat–TAR interaction,[115] several of the peripheral exocyclic substituents are amino functionalities or side chains of basic amino acids, which are likely to contribute to polar hydrogen-bonding and electrostatic interactions with RNA. Similarly, the compounds **10**, **11,** and **12**, which share a phenylfuran core scaffold (Figure 4), carry basic amino groups. The quinoxaline-2,3-dione derivative **10** has been synthesized through a combinatorial approach and identified as a ligand with low micromolar affinity for the GTPase-associated domain in 23S rRNA.[113] The molecule can perhaps mimic in part the linear peptide extension of thiostrepton, which is thought to interact with RNA in a narrow deep pocket (see above). Compounds of the diphenylfuran series[116,117] **11** and the benzimidazolyl–phenylfuran derivatives[117] **12** have been extensively studied as ligands for RNA and inhibitors of the HIV-1 Rev peptide–RRE RNA interaction. A large number of derivatives has been synthesized and their structure–activity relationships described in detail.[116,117] The most critical structural feature required for high-affinity RNA binding is the presence of cationic substituents flanking the phenylfuran core on both sides. The RNA binding site of the phenylfuranes on RRE RNA is not precisely known, but it has been suggested that a binding mode involving both partial intercalation and specific recognition of noncanonical base pairs is responsible for target recognition.[116,117]

Combined contributions from intercalation and electrostatic interactions also play a role for RNA binding of the aminoacridine derivative[118] **14** and the phenothiazine[119] **15** (Figure 4), which are both inhibitors of the HIV-1 Tar–TAT interaction. The aminoacridine **14** has been designed as a modular hybrid of an acridine intercalator and flexibly linked basic amine functionalities, and inhibits the Tar–TAT complex at concentrations in the nanomolar range.[118] The compound has later been found to disrupt as well the U1A protein–RNA complex.[120] Recognition of the largely dissimilar structures of the RRE and U1A RNA targets may reflect the somewhat promiscuous RNA affinity of the conformationally flexible aminoacridine **14**. The structurally related phenothiazine **15** has been initially identified in a computational screen for binders of the TAR RNA.[121] A three-dimensional structure model of the TAR–phenothiazine complex determined by NMR reveals that the flat aromatic ring system indeed intercalates between base pairs, and the aliphatic tail aligns itself along the shallow groove of the TAR RNA.[119]

Oxazolidinones (**16**, Figure 4) are currently the only known fully synthetic antibiotics that target rRNA of the bacterial ribosome.[122–125] While they were discovered about 20 years ago,[126] the first oxazolidinone antibiotic, linezolid (**16**, R^1=morpholino, R^2=F), has been approved for therapeutic use only recently (April 2000).[127] Meanwhile, the mechanism of action of the oxazolidinones has been elucidated, albeit their precise binding site on the ribosome remains elusive. Affinity determination and NMR experiments have shown that the antibiotics bind to 50S but not 30S

subunits.[128] The affinity of oxazolidinones to isolated 50S subunits is in the high micromolar range, which does not truly reflect their antibacterial potency.[128,129] This indicates that the binding site responsible for the antibiotic activity of oxazolidinones requires the presence of additional factors, and thus may be formed only in actively translating ribosomes. Cross-linking studies along with the mapped distribution of resistance mutations suggest that oxazolidinones target the central peptidyl–transferase loop in domain V of 23S rRNA[122–125] where they compete with chloramphenicol and lincosamides for RNA binding.[130] The antibiotics interfere with association of the initiator fMet–tRNA at the ribosomal P site and thereby inhibit the first peptide bond formation but not peptidyl transfer during elongation.[124,131] The chemical constitution of the oxazlidinone antibiotics is remarkably simple, allowing for diverse synthetic modification.[127,132] The oxazolidinone ring with an aromatic ring attached at the nitrogen and a functionalized methylene at the 5-position is found in all oxazolidinone antibiotics. Only one of the enantiomers shows antibacterial activity, although the inactive isomer can still bind to 50S subunits with low affinity. Substitution at the methylene group is most commonly by an acetamido functionality but other small acylamido substituents are tolerated as well.[127,132]

CONCLUSIONS AND PERSPECTIVE

Knowledge of RNA recognition by natural ligands is essential for attempts to design and synthesize specific RNA binders. Natural small molecule ligands for RNA and RNA–protein complexes show an amazing structural complexity and diversity of chemotypes. A multitude of functional groups allows the natural ligands to recognize their RNA targets with high specificity. Synthetic ligands attain comparable sophistication so far mostly for molecules derived by semisynthesis from natural precursors. Total-synthetic approaches towards RNA-binding ligands have focused on simplified scaffolds in order to reduce the abundance of chemically similar functional groups that complicate chemical synthesis. Reduction of structural complexity in synthetic ligands is frequently associated with loss of binding affinity and specificity for an RNA target. Remarkable exceptions are the oxazolidinones, which comprise a structurally simple scaffold substituted by a moderate number of functional groups. The oxazolidinones demonstrate that potent RNA ligands are not limited to the chemotypes found in natural ligands, which are determined by biosynthetic precursors and pathways. Thus, by harnessing the molecular diversity accessible to synthetic chemistry, novel specific RNA binders can be discovered within a molecular framework of low complexity that is amenable to established medicinal chemistry approaches.

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