Targeting RNA with Small-Molecule Drugs: Therapeutic Promise and Chemical Challenges

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ABSTRACT
Researchers' increasing awareness of the essential role played by RNA in many biological processes and in the progression of disease makes the discovery of new RNA targets an emerging field in drug discovery. Since most existing pharmacologically active compounds bind proteins, RNA provides nearly untapped opportunities for pharmacological development. The elucidation of the structure of the ribosome and other cellular and viral RNA motifs creates the opportunity for discovering new drug-like compounds that inhibit RNA function. However, further advances in understanding the chemistry and structure of RNA recognition are needed before these promises are fulfilled.

Introduction

The ever growing realization of the variety of biochemical roles of RNA in all living organisms is leading to an increasing appreciation that cellular and viral RNAs provide inviting targets to treat both infectious and chronic diseases. RNA is a validated drug target for antibacterial treatment: antibiotics in clinical practice for decades (e.g., erythromycin) bind ribosomal RNA. Furthermore, RNA is the genetic material of pathogenic viruses such as HIV or hepatitis C virus (HCV), and so it provides numerous opportunities for the discovery of new drugs to treat the devastating illnesses caused by these agents.[1] Finally, the complex functions of RNA molecules in the control of gene expression in humans provide numerous opportunities to target specific RNA structures for treating a variety of chronic and degenerative conditions.[2]

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The development of synthetically accessible analogues of RNA-binding drugs is severely limited by the lack of decades of medicinal chemistry studies dedicated to RNA and by the poor understanding of RNA recognition principles. Existing RNA-targeting drugs are complex natural antibiotics (Figure 1), and a majority of studies on RNA-binding therapeutic candidates has focused on the direct read-out of RNA sequences by antisense oligonucleotides.[3,4] As a consequence, there is no clinical example yet of the rational development of small synthetic molecules that bind a well-defined RNA molecule through the application of modern methods of drug discovery. However, natural RNAs often fold into complex structures that define unique binding sites amenable to specific recognition by small molecules. Therefore, drug designers and combinatorial chemists are seeking new chemical functions and scaffolds that are conducive to RNA binding. If the premises of these studies are realized, then a new universe of targets will become available for pharmacological intervention. Here we present a critical summary on the present status of research on RNA recognition by small molecules.

RNA as a Target for Pharmacological Intervention

RNA molecules perform many biochemical functions. The genetic information encoded as DNA in most living organisms is copied into RNA in the process of transcription. The primary transcripts (pre-mRNAs) are then processed and decoded in a highly regulated fashion by large ribonucleoprotein (RNA and protein) complexes such as the spliceosome and the ribosome. The RNA components of these machines are folded into tertiary structures that have the complexity traditionally associated with proteins. The complexity of regulation of gene expression through RNA metabolism increases with organism and tissue complexity: brain cells provide unusually abundant examples of regulation of gene expression by alternative RNA processing and small noncoding RNAs, which can potentially be targeted for pharmacological intervention.[5] Many human illnesses are associated with misfunction of RNA-processing events: at least 15% of all human genetic disorders involve aberrant mRNA processing.[6] In the vast majority of cases, these mutations map to sequences involved in controlling pre-mRNA processing.

Despite some skepticism, RNA is a well-established drug target: ribosomal RNA has long been known to be the receptor for antibiotics, and antisense oligonucleotides are being used to down-regulate gene expression. The basis of the antisense strategy is deceptively simple: sequester specific mRNA sequences with complementary oligonucleotides suitably modified to improve cellular stability and uptake and designed to form Watson−Crick

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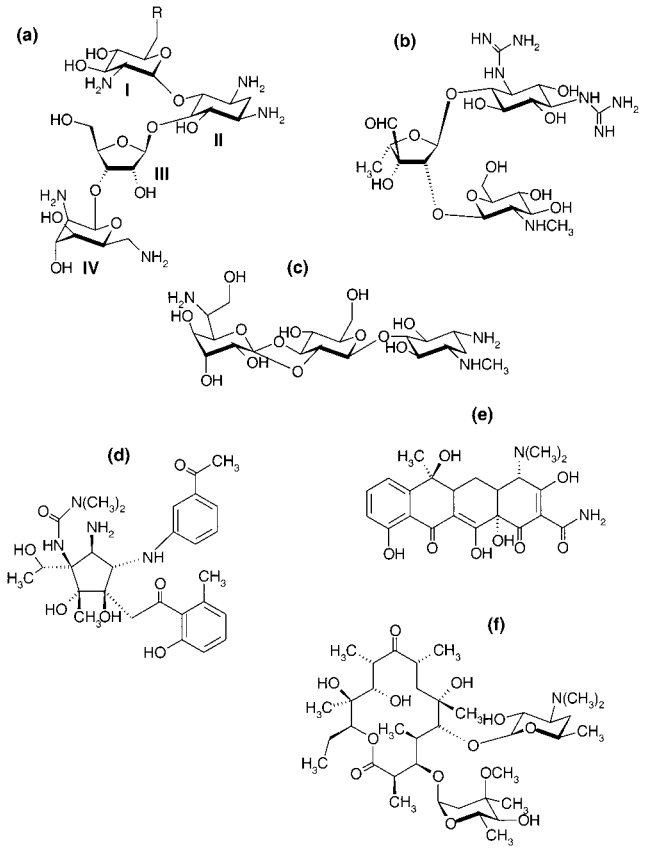

FIGURE 1. Chemical structure of selected antibiotics that bind to ribosomal RNA and inhibit bacterial protein synthesis. The aminoglycosides (a) paromomycin (R = OH) and neomycin B (R = NH_2), (b) streptomycin, and (c) hygromycin B. (d) Pactamycin. (e) Tetracycline. (f) The macrolide erythromycin. Most RNA-binding antibiotics are protonated at physiological pH, but they are represented in neutral form for simplicity.

base-pairs with the target sequence and decrease the expression of a particular gene product.[4] The first approval of an antisense drug, Vitravene, has validated the principles of the technology, and new antisense agents continue to be developed. An attractive new approach may be provided by RNA interference:[7] short double-stranded RNA duplexes have recently been shown to induce a high level of specific suppression of gene expression.[8] However, antisense or interference strategies are ripe with pharmacological problems such as unspecific protein binding and inefficient metabolic stability and cellular uptake. In addition, many mRNA sequences are not accessible to antisense agents because they are highly structured or bound by cellular proteins.

RNA molecules of viral origin provide many examples of potential drug targets for small molecules. Well-studied examples are the Rev Response Element (RRE) and the Trans Activation Responsive element (TAR) contained in mRNA molecules from the human immunodeficiency virus type 1 (HIV-1)[1,9] (Figure 2). TAR RNA is a 59-base segment of the HIV-1 genome located in the 5′ region of every HIV-1 mRNA, just downstream of the signal for the start of transcription, which adopts a highly conserved stem−loop structure. The genome of HIV-1 also encodes a regulatory protein (Tat) that interacts with TAR to

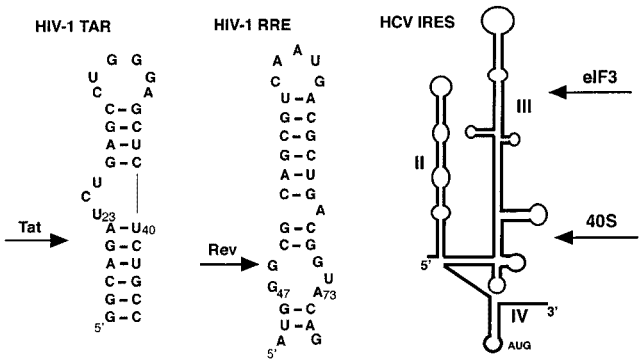

FIGURE 2. Secondary structure representations of HIV-1 TAR, HIV-1 RRE, and hepatitis C virus IRES. IRESs comprise approximately 400 nucleotides, while the HIV-1 TAR and RRE RNA stem−loops are much smaller, about 30 nucleotides. The interactions of these RNAs with HIV-1 Tat and Rev proteins, and with the eukaryotic 40S ribosomal subunit and initiation factor 3 (eIF3), respectively, are indicated by arrows.

activate transcription of all HIV-1 genes.[1,9] Agents that disrupt the Tat-TAR interaction reduce HIV-1 replication rates by interfering with the transcription of viral genes.[10−13] If specific binding to TAR RNA could be achieved, then the ensuing transcriptional block would be HIV-1 specific and would not affect cellular physiology significantly, because there is no cellular counterpart to TAR. In contrast, targeting the protein kinase required downstream of the Tat-TAR interaction for the Tat-dependent effect on HIV-1 transcription, as is certainly possible,[14] would be likely to have side effects, because nearly one-third of all human genes require this enzyme for basal gene function. Thus, HIV-1 RNA is being studied as a drug target because it provides an attractive avenue to selectivity.

Equally interesting opportunities are provided by Internal Ribosome Entry Sites (IRES, Figure 2). These are structured regions of several hundred nucleotides located at the 5′ end of the mRNA of viral pathogens such as polio, foot-and-mouth disease, and hepatitis C viruses.[15] IRESs control protein synthesis through a mechanism distinct from that of most cellular mRNAs, and their secondary structure and primary sequence are highly conserved at many (though not all) positions within different isolates of the same virus, providing again an avenue for selectivity. The structure of this vital region of viral RNA is being intensively researched, in an effort to provide the know-how necessary to develop new antivirals.[16,17]

A distinct advantage of RNA in antibacterial and (probably) antiviral treatment is that the appearance of drug resistance by point mutations in an RNA motif that is highly conserved among bacteria or different viral strains is likely to be slow. Bacteria become resistant to ribosomal RNA-binding antibiotics by exchange of genetic material encoding RNA-modifying enzymes (typically methyltransferases), drug-modifying enzymes, or enzymes that affect drug transport.[18] Therefore, if the structure of the RNA-binding drug is novel, the emergence of resistance is likely to be slower than for protein targets. This

is an important point, because resistance is the primary problem in treating bacterial[18] and viral[19] infections.

Human mRNA molecules are also being considered as potential drug targets. The sequence of eukaryotic mRNA molecules is often tissue-specific or even disease-specific due to alternative RNA processing events, providing the opportunity for hitting selectively an RNA in a desired tissue or cell line. For example, mRNAs encoding oncogenic proteins often contain unique sequences that are not found in normal cells, which can in principle be targeted for antineoplastic activity.[20,21] Another potential drug target is human telomerase, a complex ribonucleoprotein involved in chromosome maintenance that is selectively active in cancer cells: its RNA component is being investigated as a potential target for new antitumor agents.[22]

The Bacterial Ribosome Is a Fully Validated but Largely Unexploited Target for Developing New Antibiotics

Ribonucleoprotein complexes represent inviting targets for pharmacological intervention. First, there are differences between pathogenic organisms and humans; second, their structures contain well-defined binding sites composed of both RNA and proteins. An ideal example is provided by the ribosome, the ribonucleoprotein where the genetic information encoded by mRNA is translated into a protein sequence, comprising two subunits of different size, denoted 50S and 30S. The bacterial ribosome has been known as the receptor for antibiotics blocking protein synthesis since the discovery of streptomycin in the 1940s, but new antibacterials are urgently needed to overcome the problem of drug resistance that severely limits the effectiveness of the current arsenal of antibiotics.[18]

Many antibiotics bind ribosomal RNA rather than its associated proteins.[23] The atomic details of the interaction of a class of antibiotics, the aminoglycosides (Figure 1a–c), with ribosomal RNA were first revealed by pioneering NMR work.[24,25] An extraordinary breakthrough was achieved last year with the determination of the structures of both bacterial ribosome subunits by X-ray crystallography,[26,27] which have provided undreamed-for information for the design of new ribosome-targeted antibacterial drugs. There are not yet structural data available on antibiotics binding to the large subunit (where macrolides like erythromycin bind). However, two crystallographic studies of the complexes of the small (30S) subunit with antibiotics (streptomycin, paromomycin, and spectinomycin;[28] tetracycline, pactamycin, and hygromycin B[29]) have provided remarkable insight into the mechanism of action of these drugs. The 30S subunit has a crucial role in "decoding", i.e., matching the sequence of the mRNA with its corresponding aminoacylated tRNA, and it also contributes to the translocation of the tRNA and associated mRNA during elongation of the polypeptide chain. Antibiotics targeting the 30S subunit affect one of these two functions. For example, tetracycline (Figure 1e) primarily binds the aminoacyl tRNA site (called the A-site) and ejects the aminoacylated tRNA from the ribosome through steric clash[29] (Figure 3a,b). The aminoglycosides paromomycin and neomycin B (Figure 1a) bind near the decoding region, in helix 44, and flip out two adenine bases implicated in decoding, thus reducing accuracy during protein synthesis[28,30] (Figure 3a,c). Streptomycin (Figure 1b) causes the same effect by binding to four different RNA regions in the decoding site.[28] This network of interactions stabilizes a miscoding-prone form of the A-site and impedes the switch to the restrictive state through changes in packing of RNA helices.

Mechanisms of RNA Recognition by Small Molecules

The diverse biological functions of RNA are linked to its capacity to form complex three-dimensional structures: in this respect, RNA is similar to proteins. However, it is chemically less diverse, since proteins are composed of 20 different amino acids as opposed to just four different types of nucleotides. The purine and pyrimidine bases of RNA are nonetheless functionally rich moieties susceptible of direct readout via specific hydrogen bonds and stacking interactions. The questions of how structurally and chemically diverse RNA motifs are, and if they can be recognized with small molecules with the same specificity as proteins, are essential considerations for drug development. The recent work on the ribosome RNA has addressed some of these questions satisfactorily, but uncertainties still remain for other RNAs that do not have the elaborate three-dimensional architecture of the ribosome.

Ribosomal RNA is mainly composed of irregular double-helical stems and loops organized in a complex tertiary structure. Clefts between the different helices and loops form well-defined binding sites for small-molecule drugs. The mechanisms of ribosomal RNA recognition by drugs are diverse among different compounds and compound classes. For example, tetracycline is a neutral polycycle with polar functionalities on one side (Figure 1e), which binds two sites within the 30S ribosomal subunit. In both sites, the majority of interactions are established between the polar groups of the drug and the sugar–phosphate backbone of nearby RNA helices and loops[29] and weak or no stacking interactions are observed (Figure 3b). In contrast, pactamycin (Figure 1d) mimics an RNA dinucleotide, and the two aromatic rings of the drug establish stacking interactions between themselves and with a nearby stem–loop.[29] Aminoglycoside antibiotics are positively charged, flexible molecules containing several aminosugar rings linked in a linear array (Figure 1a–c) that establish polar contacts with RNA backbone and base groups in the major groove (Figure 3c). Paromomycin and neomycin B (Figure 1a) displace two adenine bases out of helix 44[24,25,28] (Figure 3a). Hygromycin B (Figure 1c) binds to a different site within helix 44, but in this case no base displacement is observed, and RNA recognition is mediated almost exclusively by sequence-specific reading of individual nucleobases via hydrogen bonding.[29]

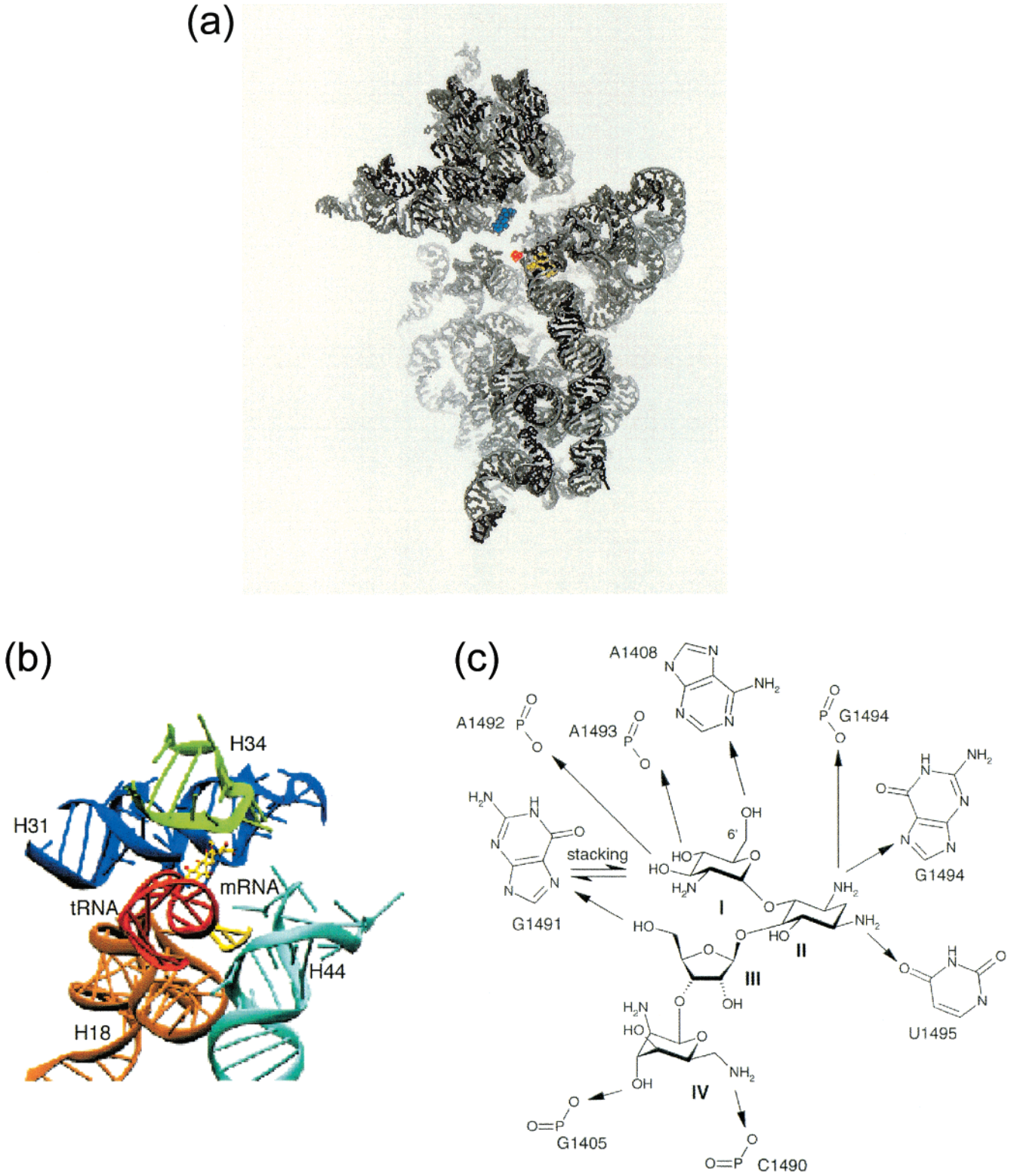

FIGURE 3. (a) View of the bacterial 30S ribosome subunit (as seen from the 50S subunit) with tetracycline (blue) and paromomycin (yellow) bound in the aminoacyl tRNA site. Two adenine bases flipped out by paromomycin are shown in red. (b) Tetracycline binding site in the 30S subunit. The tRNA (in red) and mRNA (in yellow) molecules are modeled. (c) Schematic representation of the interactions established between paromomycin and RNA residues in helix 44 of ribosomal RNA.

In less complex RNA structures, small-molecule binding sites are generally more flexible and exposed to the solvent. RNA aptamers, oligonucleotides selected using in vitro genetic methods on the basis of their ability to bind tightly and specifically to a variety of small molecules, provide an interesting case study in RNA recognition.[31−33] The analysis of aptamer−ligand complexes demonstrated that the ability of RNA to bind small molecules tightly and

specifically resides in irregular structures stabilized by numerous intramolecular interactions, in addition to contacts with the ligand. These and other studies have shown that drug binding to RNA occurs frequently by induced fit, as observed for the interaction between the HIV-1 TAR RNA regulatory element[34] and Tat-derived peptides and organic molecules.[10,12,35]

If small molecules are likely to exploit the plasticity of RNA structure to define a suitable binding pocket, what is the appropriate structure to target? The conformational landscape of many RNA molecules is rugged, with many conformational states of comparable energy and with relatively low energy barriers separating them. Small-molecule ligands can affect the population of different states and induce an entirely new conformation in the RNA target. RNA flexibility presents obvious problems for structure-based drug design, but it increases the opportunity for therapeutic intervention, allowing for allosteric effects in addition to direct competition.[36] Allosteric regulation can be obtained by forcing the RNA into a conformation that is unsuitable for its function, as observed for the binding of neomycin B to HIV-1 TAR RNA.[36] Alternatively, a therapeutic effect can also be obtained by stabilizing the RNA into a single conformation that is incapable of switching between different conformers, as is likely to be the case for antibiotics that bind the GTPase center of the ribosome.[37]

Since RNA molecules are negatively charged, electrostatic interactions are critical for binding. Most known RNA-binding ligands are cationic molecules, and RNA affinity and discrimination are modulated by the interplay of nonspecific electrostatic forces, which are critical for affinity,[38] and specific interactions. The flexible and polycationic aminoglycoside antibiotics preferentially bind to prokaryotic ribosomal RNA (Figure 3c), but they also bind to a variety of unrelated RNAs, including the TAR and RRE HIV-1 RNA motifs,[36,39] several ribozymes,[40] and human mRNAs.[20,21] Thus, electrostatic interactions are a double-edged sword: they boost affinity, but at the price of reduced specificity and inefficient cellular uptake.

High-Throughput Screening Methods Are Also Applicable to RNA

There are some practical advantages to RNA as a drug target, when compared to proteins. RNA oligonucleotides are more easily prepared by chemical or enzymatic synthesis than proteins and are very often soluble. Compared to proteins, RNA regulatory elements tend to be smaller: RNA structure is mainly driven by base-pairing, so well-designed oligonucleotide models often retain the same local structure and interactions as in intact RNAs.[24] These features often allow the straightforward investigation of RNA−ligand interactions.

In the past few years, the fundamental approach to drug discovery, high-throughput screening of proprietary collections of chemicals or combinatorial libraries, has therefore been applied to several RNAs. Fluorescence-based techniques are currently the most popular screening method. Phosphoramidite chemistry allows the synthesis of RNA molecules labeled with appropriate fluorescent dyes, and ligand binding to RNA can be identified by one of several fluorescence-based techniques.[13] For example, a fluorescent antibiotic can be used in a competition assay to determine the binding of new ligands by a displacement assay.[21,41] A recent method of fairly high throughput is based on surface plasmon resonance, which relies on immobilizing a receptor on a surface and monitoring changes in the optical properties of the surface−liquid interface that occur when a ligand binds the immobilized molecule.[39] Implementation of the method requires the chemical modification of the receptor for linking it to the surface, which can easily be accomplished by preparing biotinylated oligonucleotides, and allows quantitative measurements of binding affinities and kinetics.[20,39,42,43] Another addition to the arsenal of tools for high-throughput screening of RNA−drug interactions is mass spectrometry. Recent developments in instrumentation have increased the mass range and decrease the amount of sample required, to the point of allowing analysis of noncovalent complexes. This method has the advantage of not requiring any modification of the ligand or the RNA and can be easily applied to the analysis of large compound libraries because it is eminently suitable for multiplexing.[11,44]

NMR spectroscopy can also be used to monitor the binding of ligands to macromolecules, to identify binding sites, and to design new lead compounds.[45] Methods that rely on the NMR observation of the signal of the ligand rather than the receptor allow the direct identification of specific ligand binding and have no molecular weight limitations. When a ligand binds to a receptor with higher molecular mass, its transverse relaxation time decreases while the sign and magnitude of its NOEs change, and its translational diffusion coefficient diminishes. All these changes can be detected by simple 1D or 2D experiments in mixtures of 5−10 compounds and can be used to screen even very large libraries of tens of thousand of compounds.[45] Application of these methods to RNA is being pursued both in the biotechnology industry and in academic laboratories.

Computational Methods for Structure-Based Drug Discovery

If the structure of a receptor is known, docking programs can be used to calculate theoretical binding affinities of large pools of drug candidates in a fast and economic way, and can also be useful for de novo ligand design. These computational searches are routinely used for protein drug design[46] and have been applied to double-helical RNA and the HIV-1 TAR system with some success.[47,48] The search algorithms are based on fitting the ligands on the binding site by rigid or flexible docking. The interaction is then evaluated through "scoring functions", measuring parameters such as van der Waals interactions, the reduction in exposed surface area, and the number of hydrogen bonds.[46] However, if the conformation of the

RNA is flexible or varies upon drug binding, as previously mentioned, then the search process can be compromised. More structural and thermodynamic information on RNA−small molecule interactions is clearly needed to develop more effective computational tools that are suitable for the RNA environment.

The Chemistry of Small Molecules That Bind RNA

The medicinal chemistry of RNA-binding drugs is in its infancy because, with the exception of the bacterial ribosome, RNA has only recently been considered as a valuable source of drug targets. Naturally occurring antibiotics that bind ribosomal RNA are difficult to modify, and, as a consequence of the lack of many years of medicinal chemistry efforts, few functional groups and scaffolds are available as building blocks to develop new RNA-binding entities. However, the recent discovery of a new class of antibacterial drugs reinforces the importance of RNA as a drug target: the oxazolidinones (Figure 4a) are active against a wide variety of Gram-positive bacteria and are ribosomal RNA-binding drugs.[49]

Because they bind many other RNAs, including the HIV-1 TAR and RRE,[36,39] aminoglycoside antibiotics have been used for the development of new drugs, although these drugs have limited specificity and undesirable side effects because they bind promiscuously, probably due to their polycationic nature (Figure 1a−c). Synthetic analogues of aminoglycoside drugs have been designed in order to define their ribosomal RNA-binding pharmacophore. Substitution of ring IV (Figure 3c) with simpler amino groups connected through a flexible linker reduced RNA binding in vitro, although it had only a small effect on antibacterial activity.[42] Combinatorial libraries based on an aminoglucopyranoside ring were also tested on a model 16S ribosomal RNA fragment.[43] These compounds simplify the structure of aminoglycosides by embedding the 1,2- and 1,3-hydroxyamine motifs found within the natural drugs and involved in RNA phosphate and base recognition (Figures 3c and 4b). Some of the resulting molecules had affinity in the micromolar range, similar to the parent antibiotics. Even simpler 1,3- and 1,2-aminols (Figure 4c), designed on the basis of the same principle, were found to compete with aminoglycosides in binding to 16S RNA fragments.[41] These results are encouraging because they demonstrate that it is possible to design chemically accessible, drug-like molecules while retaining high RNA-binding affinity.

The HIV-1 TAR and RRE RNA motifs (Figure 2) have been used as testing grounds for discovering scaffolds and functional groups suitable for RNA binding. A library of mimetics of the aminoglycoside neomycin B was synthesized using rapid combinatorial synthesis in an effort to discover new inhibitors of the interaction between the HIV-1 RRE RNA and Rev protein[50] (Figure 4d). Some of the resulting products blocked the RRE-Rev interaction with IC_{50} values in the micromolar range. Tetracationic compounds consisting of two aminoalkyl substituents

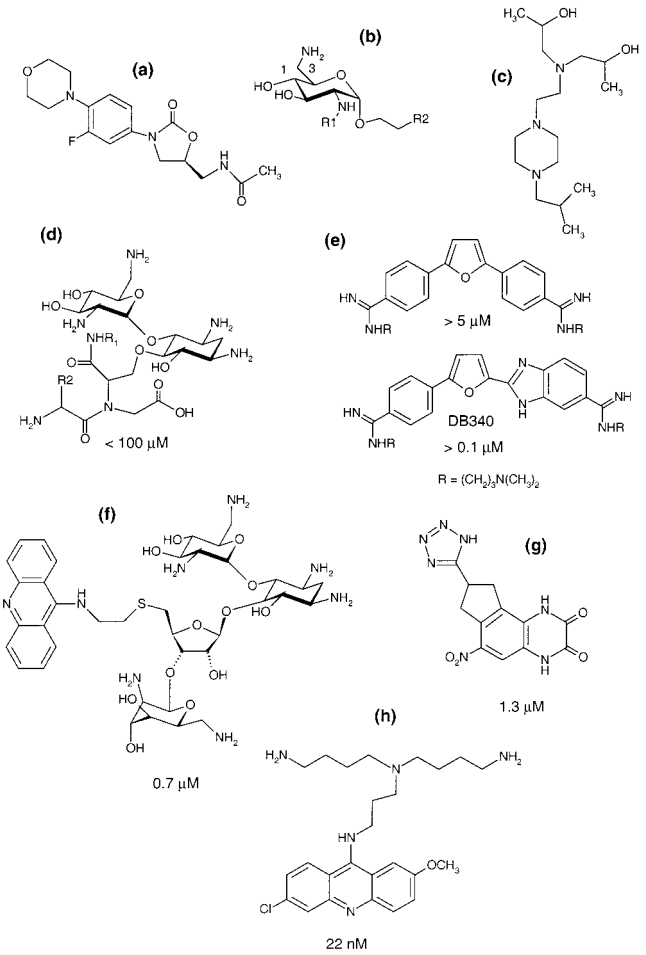

FIGURE 4. Chemical structure of new RNA-binding drugs. (i) Compounds targeting ribosomal RNA: (a) the oxazolidinone linezolid; (b) the 1,3-hydroxyamine aminoglucopyranoside combinatorial library; (c) a 1,2-aminol molecule. (ii) HIV-1 RRE-Rev inhibitors: (d) neomycin-mimetic combinatorial library; (e) diphenylfurans; (f) a neomycin−acridine conjugate. (iii) HIV-1 TAR-Tat inhibitors: (g) quinoxaline-1,2-dione; (h) aminoalkyl-linked acridine. For (ii) and (iii), the compound concentrations required to dissociate 50% of the RRE-Rev and TAR-Tat complexes, respectively, are indicated.

linked to a central diphenylfuran aromatic ring (Figure 4e) were found to bind to RRE RNA and inhibit the Rev-RRE interaction at concentrations of 0.1−5 μM, 10-fold lower than neomycin B.[51] The structure of these agents is reminiscent of DNA minor groove binders such as netropsin. In fact, one of these compounds (DB340) binds as a dimer in the minor groove of the RRE internal loop.[52]

Combinatorial libraries based on cationic functionalities were designed to target HIV-1 TAR RNA, and some compounds showed submicromolar TAR-Tat inhibition values.[53] Several TAR-Tat inhibitors were also discovered through the systematic search of the Parke-Davis proprietary library, and a quinoxaline-2,3-dione molecule (Figure 4g) was found to bind to the bulge loop.[11] Peptides and peptidomimetic molecules have also been shown to be active in inhibiting the HIV-1 TAR-Tat interaction.[10,13,54]

The synthesis of a series of compounds consisting of a heterocyclic aromatic system (acridine or naphthalimide) linked to a flexible cationic aminoalkyl moiety resulted

in the discovery of a new class of inhibitors of the HIV-1 Tat-TAR interaction.[12] The most active compounds in this class competed with the TAR-Tat interaction in vitro at nanomolar concentration (Figure 4h) and blocked HIV-1 replication with an IC_{50} of 1.2 μM by binding to the major groove of the TAR bulge loop[12] (Figure 2). A related and recently synthesized acridine−neomycin conjugate (Figure 4f) inhibits the RRE-Rev interaction with an IC_{50} of 0.7 μM by binding to the RRE internal loop.[55] Both acridines and aminoalkyl-linked naphthalimides bind to double-helical DNA as intercalators, but these compounds are interesting because they suggest that recognition of unpaired bases in RNA can be mediated by stacking interactions with drug chromophores, without classical intercalation.[12]

The results of these studies are encouraging, but further efforts in synthetic chemistry and detailed structural studies of the complexes between small molecules and RNA are still sorely needed. Specificity appears to be a critical issue, since many of the molecules presented here interact with multiple RNAs. An avenue to selectivity yet to be explored is targeting of *interfaces* between proteins and RNA.

Conclusions

The considerations of the first part of the present Account indicate that opportunities abound to target RNA regulatory elements within bacterial, viral, and human RNA molecules. The recent structures of the ribosome and the marketing of oxazolidinones have already had a significant impact on the pharmaceutical industry, by highlighting the opportunities for the discovery of novel RNA-binding antibiotics. New antibiotics will likely be found in the next few years that exploit the prokaryotic ribosome as a drug target, and they will probably be developed through a combination of high-throughput screening, combinatorial, and structure-based methods. These programs, now ongoing in several pharmaceutical and biotechnology companies, will also generate the information on chemistry and molecular recognition needed to solve the more difficult problem of recognizing the simpler structural features present in mRNAs of viral or human origin. None of the studies described in this Account has yet resulted in the successful preclinical development of sustainable antiviral or antibacterial leads. When the technological challenges highlighted in this Account are overcome, new avenues will become available to treat both infectious and noninfectious diseases by interfering with the function of RNA and its complexes with regulatory proteins.

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