RNA as a target for small-molecule therapeutics

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Natural antibiotics that specifically target RNA components of the bacterial ribosome set precedence for RNA-directed small-molecule drugs. Structured domains of functional RNAs in bacteria and viruses have thus attracted attention as targets for the discovery of novel anti-infectives. Whereas a growing number of RNA-directed ligands have been reported in the literature, creating synthetic molecules that combine high affinity with selectivity for a specific RNA target remains a key challenge. Ongoing discovery efforts towards this goal, combined with the increasing knowledge of the factors that govern small-molecule recognition of RNA folds, will prepare the ground for the development of novel RNA-targeted therapeutics.

Keywords: aminoglycosides, HIV, ribosome, RNase P, X-ray crystallography


1. Introduction

The discovery and development of antibiotics represent one of the major advances of the 20th century. These low molecular weight drugs of natural, semisynthetic or synthetic origin have successfully combated, and in certain cases eradicated, life-threatening infectious diseases. Unfortunately, the past few decades have brought viral and bacterial infections back to a focal point. In addition to the appearance of 'new' viruses there has been a resurgence of previously known infectious diseases, as well as an emergence of resistant mutants of recognised microorganisms.

In search for new approaches to combat human pathogens, viral and bacterial RNA sequences have recently emerged as intriguing therapeutic targets. Although the fundamental roles of DNA and proteins as 'information storage' and functional biomolecules, respectively, have not changed since the original proposal of the central dogma of molecular biology, fascinating new functions have been discovered for RNA. Far from being a passive mediator of genetic information, RNA is now appreciated to be a versatile functional biomolecule closely involved in numerous cellular processes. Key events in bacterial and viral reproductive cycles that are mediated by RNA or RNA–protein interactions have therefore become the focus of intense investigations.

Despite its limited 'vocabulary', the ability of RNA to fold into intricate three-dimensional structures facilitates specific communication with large biomolecules and, in principle, generates potential binding pockets for small molecules [1-3]. Although several motifs for RNA-binding proteins have been identified, very few RNA sequences have been validated as therapeutically meaningful targets. This shortcoming is likely to be alleviated as the understanding of the biology and molecular recognition features of RNA advances. Recent findings demonstrating specific interactions between low molecular weight metabolites and mRNAs related to their biosynthetic pathways [4,5] illustrate that nature has been taking advantage of RNA–small-molecule interactions to regulate important biochemical transformations at the RNA level. In addition to the clarification of basic biochemical pathways, these exciting discoveries provide the context for the search for
synthetic ligands that target biologically important RNA sequences.

Our understanding of RNA molecular recognition by small molecules is currently limited by the relatively biased chemical diversity of natural RNA ligands. By and large, and not too surprisingly, many of the naturally occurring RNA binders known to date target the ribosome [6,7]. This huge cellular machine possesses several vulnerable spots that are elegantly exploited by far smaller natural products such as the aminoglycoside and macrolide antibiotics. Their efficacy is yet to be matched by artificial synthetic RNA ligands. With some exceptions, most drug-like small molecules designed to target RNA sites exhibit lower affinity and, more importantly, lower target specificity. Although sometimes subtle and rarely emphasised in the literature, achieving desirable level of selectivity is, at this point, the most challenging aspect afflicting the search for synthetic RNA binders.

Due to efforts by both academic and industrial laboratories over the past decade, RNA has been slowly gaining its place as a viable target for therapeutic intervention. A previous review by Griffey and Swayne [8] outlined the prospects and promise of RNA-targeted therapeutics. Here, an updated view of the field is provided and recent efforts to develop small organic molecules as specific binders of bacterial and viral targets are emphasised.

2. Bacterial RNA targets

2.1 The bacterial ribosome

Bacterial ribosomes contain the only validated RNA targets for which approved drugs are currently available. Indeed, a significant number of antibiotics classes that bind to ribosomal RNA, and thereby interfere with bacterial protein synthesis, are known. The interest in the ribosome as a target for the discovery of new antibacterials has been spurred over the last five years by the structure determination of whole bacterial ribosomes, subunits, domains and ligand complexes thereof. A patent has been obtained for the crystallisation of the bacterial ribosome [201], and applications have been filed for the determination and uses of the three-dimensional structures [202-206]. As several reviews have comprehensively outlined the chemical diversity of ribosome-directed antibiotics [6,7,9-11], some recent pertinent patents and publications will be described in brief.

2.1.1 The ribosomal decoding site

The A- or decoding site within bacterial 16S ribosomal RNA (rRNA) is the target for many natural aminoglycoside antibiotics that, upon binding, interfere with translational fidelity. The natural products suffer from low bioavailability and toxicity, both linked to their polycationic character. Over the last decades, numerous attempts have been made to develop improved aminoglycoside analogues. Recently, groups at Wayne State University [12,13], the Technion [14], Isis Pharmaceuticals [15,16], Optimer Pharmaceuticals [17] and Anadys Pharmaceuticals [18-22] have published antimicrobial decoding-site ligands based on aminoglycoside derivatives and mimetics that were, in part, designed using structural information of the bacterial RNA target. Mobashery at Wayne State University has described the discovery of a 1,6-disubstituted neamine ligand of the decoding site (1, Figure 1) [12] and, in collaboration with colleagues at Ribotargets, determined the three-dimensional structure of the compound bound to RNA [13]. Crystal structures of aminoglycosides bound to decoding site RNA have revealed the key role for target recognition of the 2-deoxystreptamine (2-DOS) moiety (2, Figure 1) [23], the common core scaffold of the natural aminoglycosides. Efforts at Isis, Optimer and Anadys have explored extensively the antimicrobial activity of synthetic 2-DOS derivatives and analogues [15-19]. Patents on 2-DOS-based synthetic aminoglycosides were pursued by the Isis group [207,208]. Replacement of the 2-DOS moiety by heterocyclic scaffolds has yielded a novel series of piperidine- and azepanoglycosides as aminoglycoside mimetics described by the Anadys group (3 and 4, Figure 1) [21,22].

The sugar components of aminoglycosides have been used by the Isis group to synthesise linked carbohydrates, which showed low micromolar affinity for the decoding site RNA [24]. Optimer Pharmaceuticals has filed a patent application on novel anti-infectives focusing on the linking and modification of aminoglycoside saccharide scaffolds [17,209]. Aminoglycoside dimers that bind to the bacterial decoding site and also act as inhibitors of modifying resistance enzymes were disclosed in a publication and covered in a patent application filed by the Scripps Research Institute [25,210]. The Technion group has synthesised antibacterial pseudo-pentasaccharide aminoglycosides by linking an additional sugar moiety at the 5'-position of the natural antibiotic neomycin B (5, Figure 1) [14]. A laboratory at the Taiwan Genomics Research Center has published a novel approach for regioselective glycosylation of neamine to synthesise aminoglycoside derivatives [26].

The natural abundance of aminoglycoside ligands confirms the significance of the bacterial decoding site as a validated target for antibacterials. Thus, attempts have been made to discover novel non-aminoglycoside ligands that avoid the intricacies involved with synthesis around the complex chemical structures of the natural antibiotics. Screening campaigns directed at the bacterial decoding site have been reported by groups at Isis, Abbott Laboratories and Ribotargets (now Vernalis Ltd). The Isis group has used their proprietary mass spectrometry (MS) assay [8] to identify a decoding site-binding benzimidazole [27]. The lead compound was optimised using an MS-guided technique [28], eventually yielding a piperidine-benzimidazole derivative with an affinity of 60 μM for the bacterial tRNA target (6, Figure 1).

The Abbott group has published a nuclear magnetic resonance (NMR)-based approach to identify, among 10,000 tested compounds, 2-aminoquinoline and 2-aminopyridine derivatives as decoding-site ligands with micromolar affinities [29]. The RNA complex of the tightest binder, N-(aminoethyl)-4-methyl-2-aminopyridine (7, Figure 1) with a
K₄ value of 3 μM for the decoding site, was characterised by NMR and modelled using NOE constraints. The authors speculate that compounds of the 2-aminoquinoline and pyridine series likely also bind to other RNA targets, although no data are reported. A similar application of NMR-based screening of molecular scaffolds against an RNA target, the P4P6 domain of a self-splicing Group I inttron, has been described by groups at the Scripps Research Institute and Vertex Pharmaceuticals [30].

The Ribotargets group has used their proprietary RiboDock program to screen in silico ≈ 890,000 compounds for potential ligands of the bacterial decoding-site RNA [31]. Selected hits were tested for decoding site binding in a proprietary fluorescence resonance energy transfer (FRET)
assay [211,212] that monitored displacement of a fluorescence-labelled aminoglycoside ligand. The identified binders were mostly aromatic compounds carrying positively charged groups, among them several substituted quinolines and quinolones. Similar to the Abbott approach, intermolecular NMR NOE data were used to characterise the ligand–RNA complexes. The best compound, a 4-hydroxy-2-quinolone-3-carboxylic acid amide (8, Figure 1), had a $K_D$ value of 17 μM for binding to the decoding site as determined by the FRET assay. The authors suggest a specific interaction of the compound with the unpaired A1408 of the decoding-site RNA. Data on binding selectivity for the ribosomal versus other RNA targets is not reported.

In addition to the generic screening approaches for RNA described above and in the patent literature [213-215], a method based on microarray-immobilised aminoglycosides has been described by a group at the ETH in Zurich [32], and target-specific fluorescence assays have been published by laboratories at the University of New Jersey [33] and at Anadys Pharmaceuticals [34]. These fluorescence assays exploit the intrinsic conformational flexibility of two unpaired adenine residues within the decoding site, and are based on RNA constructs in which one of the adenines is replaced by a fluorescent base, such as 2-aminopurine. Binding of decoding site-specific ligands, including natural aminoglycoside antibiotics, triggers a conformational change within the unpaired bases that impacts fluorescence yield of the modified base. The Anadys group has used this assay to establish structure-affinity relationships for designed aminoglycoside mimetics [34].

2.1.2 Other ribosomal targets and ligands

Over the last 5 years, crystal structures of ribosomal subunits have revealed the binding sites of important antibiotic classes, including tetracyclins, macrolides (9, Figure 2) and ketolides (10, Figure 2) [6,7,10]. Co-crystal structures of oxazolidinones (11, Figure 2), the only known fully synthetic antibiotics that target the bacterial ribosome, have not been published yet, leaving their precise binding site open to debate. While crosslinking studies indicate interaction with the Exit (E) site, close to the binding site for protein L1 [35], biochemical and NMR data suggest that the oxazolidinones target rRNA at the peptidyl-transferase site [36,37], similar to the macrolides and chloramphenicol. Perhaps the two clinically most important new classes of ribosome-targeted antibiotics are the oxazolidinones (linezolid, 11, Figure 2) and ketolides (telithromycin, 10, Figure 2) which were approved in 2000 and 2001 (in Europe), respectively. Reflecting the active antibacterial discovery efforts by many companies, a large number of patents have been published that cover novel oxazolidinones [216-228], macrolides [229-246], streptogramins [247], and tetracyclins [248-250]. Whereas the disclosed series seem to be the results of traditional medicinal chemistry or genetic engineering programs, structural information on the binding sites of the ligands at their rRNA targets will be increasingly important in the design of novel antibiotics in these classes. It has been recognised, for example, that efficacy of macrolide and ketolide derivatives is dependent on the conformational rigidity of the lactone core scaffold, which can now be rationalised in light of the ribosomal complex crystal structures. In potent macrolides, the lactone ring adopts a folded-out conformation that projects most polar groups towards one face of the molecule, whereas the opposite side of the macrocycle is hydrophobic [38]. Conformationally locked macrolides that carry a covalent bridge across the lactone ring (12, Figure 2) have been patented by Enanta Pharmaceuticals [239,243].

Structural information on the bacterial ribosome also facilitated the discovery of a second class of macrocyclic antibiotics, the thiazole-peptides such as thiostrepton and micrococin. These macrocycles interact with 23S rRNA at the GTPase-associated domain of the 50S ribosomal subunit and shut down GTP-dependent reactions during translation. Whereas, in the past, the water-insolubility of thiostrepton has prevented crystallographic studies on the thiazole-peptides, NMR and biochemical studies at Ribotargets have led to the construction of a three-dimensional model of thiostrepton bound to its RNA target [39]. Insight from these investigations, in combination with an assay that measured target-specific methylation by a thiostrepton-resistance enzyme, provided the basis for the discovery of synthetic thiostrepton fragments [40]. Compounds resulting from this effort (13, Figure 2) showed interaction with the RNA target and inhibition of methylation in the high micromolar range, but no antibacterial activity. Some of the most potent inhibitors of RNA methylation were nonspecific ligands of the RNA target. Griffey and co-workers at Isis have used their proprietary MS-based assay method to screen synthetic peptide libraries for ligands of the thiostrepton-binding site [28]. They were able to identify thiostrepton mimetics (14, Figure 2) that bound to the target RNA with low micromolar affinity and inhibited *in vitro* bacterial translation with similar potency. Antibacterial testing has not been reported for these compounds. No patent activity has followed from the work on thiostrepton mimetics at Isis or Ribotargets.

Insight into the topological proximity of ligand binding sites in the bacterial ribosome, together with a better understanding of the molecular recognition of RNA, have been used to design translation inhibitors based on conjugates of known RNA binders. A group at the Korea Institute of Science and Technology has synthesised RNA-targeted ligands by linking the decoding site binder neomycin B with chloramphenicol or an oxazolidinone, which recognise the peptidyl-transferase site [41]. The compounds were tested for their affinity for various RNA targets; however, not including ribosomal RNA. A patent application has been filed for the neomycin–chloramphenicol conjugates as potential antibacterials or antivirals [251]. Conjugates of aminoglycosides and the basic amino acid arginine that were previously synthesised as ligands of HIV transactivating region (TAR) RNA (see section 3.1.2) were found to also interact with ribosomes and inhibit translation, albeit without specificity for the bacterial
Figure 2. Structures of ligands for other ribosomal RNA targets (9 – 16) and RNase P (17). 9) Erythromycin, a natural macrolide; 10) telithromycin (Ketek®, Aventis); 11) linezolid (Zyvox®, Pharmacia/Pfizer); 12) 6,11-bridged macrolides by Enanta Pharmaceuticals [239,242]; 13) synthetic thioestrepton fragment by Ribotargets [40]; 14) synthetic thioestrepton mimic by Isis Pharmaceuticals [28]; 15) fluoroquinolone translation inhibitor by Abbott Laboratories [45]; 16) 14-membered macrocyclic translation inhibitor by Isis Pharmaceuticals [47]; 17) aromatic bis-guanylhydrazone RNase P inhibitor by Message Pharmaceuticals [258,259]. For discussion, see text.
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2.3 Ribonuclease P

Maturation of the 5’-termini of precursor tRNAs is achieved by ribonuclease P (RNase P). The bacterial RNase P consists of a catalytic RNA of several hundred nucleotides and a protein cofactor. Certain antibiotics that act on rRNA, including the aminoglycosides, have been shown to also bind to the RNA component of bacterial RNase P and thereby block the maturation of precursor tRNAs [52]. Based on the significant divergence of the RNase P enzymes of eubacteria and eukaryotes, bacterial RNase P is considered a viable target of antibiotics discovery [53]. In succession to older patent literature, applications have been filed recently to cover the RNase P RNA targets of Staphylococcus pneumoniae and Staphylococcus aureus [256,257]. Aminoglycoside–arginine conjugates have been shown to inhibit bacterial RNase P [54], albeit with low specificity, as these conjugates were also inhibitors of translation (see section 2.1.2) and HIV Tat–TAR interaction (see section 3.1.2). Message Pharmaceuticals has applied for patent coverage of small-molecule inhibitors of bacterial RNase P as antibacterials [258,259]. Among the claimed compounds are aromatic bis-guanyldihyrazones (17, Figure 2) that showed antibacterial activity at low micromolar concentrations.

3. Viral RNA targets

3.1 RNA targets of HIV

Noncoding regulatory domains of the HIV genome were among the first RNA structures apart from bacterial tRNA that were recognised as viable targets for small-molecule therapeutics [55-57]. Since then, numerous studies have explored the interaction of the HIV TAR RNA and the Rev-response element (RRE) RNA with natural and synthetic ligands. Earlier work on these targets was summarised in a number of reviews [8,9,58-60]. Here recent developments over the last few years are focussed on.

3.1.1 HIV Rev-response element

The HIV Rev protein binds to the RRE domain of viral primary transcripts and induces export of partially spliced and unspliced mRNA for the translation of late-stage viral proteins. The aminoglycoside neomycin B (Figure 1) has been shown to bind to the RRE–RNA and inhibit the interaction with Rev as well as HIV replication in cells [55,56]. Assays have been developed that measure the Rev–RRE complex disruption by small-molecule inhibitors. Tor et al., at the University of California San Diego (UCSD), have used methods based on the displacement of a fluorescence-labelled Rev peptide to measure binding of Rev–RRE inhibitors [61]. The UCSD group has synthesised and tested a wide range of aminoglycoside derivatives, including dimers and intercalator conjugates such as neomycin-acridine (18, Figure 1), for their binding specificity for the RRE RNA target [61-63]. Their work...
underlines the importance of specificity studies on RNA-directed ligands, many of which display promiscuity towards different RNA targets. A systematic investigation of the binding of aminoglycoside derivatives to RRE RNA illustrates the key challenge for the development of drugs for RNA targets [62] where an inverted relationship between general RNA affinity and target specificity is exhibited by most currently known RNA binders. The exploration of phenanthridinium derivatives as RRE ligands by Tor yielded a 3,8-bis-urea-ethylendiamine-ethidium derivative (19, Figure 3) that had an RRE RNA affinity in the submicromolar range and a selectivity of 2000-fold for the RRE target versus non-specific nucleic acids [63]. Increased target-specificity combined with higher affinity for the RRE RNA was observed for guanindinoglycosides (20, Figure 3) that were obtained by modification of natural aminoglycosides by guanylinylation of their amino groups [64]. Unlike aminoglycosides, the guanindinoglycoside derivatives showed efficient uptake by eukaryotic cells, perhaps by an active transport mechanism [65], and inhibition of HIV replication in cell culture superior to the parental aminoglycosides [66]. A patent application has been filed for guanindinoglycosides and their use as antivirals in HIV infection [260]. Patent coverage has also been claimed for aminoglycoside–chloramphenicol conjugates [251], which were reported to display enhanced specificity for the RRE target [41]. A fluorescence screening method for RRE ligands based on 2-aminopurine-labelled RNA constructs [67], in combination with NMR observation of proton chemical shifts during compound titrations, has been used to discover profilavine (21, Figure 3) as a ligand that binds RRE RNA with submicromolar affinity [68]. Further analysis revealed that profilavine binds in a specific non-intercalative mode with a stoichiometry of 2:1 to a single site on the RRE RNA. It has been speculated that antiviral agents can potentially be made by linking two profilavine moieties, eventually yielding an RRE-specific ligand that might avoid promiscuous intercalation of individual profilavine into other cellular nucleic acid targets [68]. Other screening methods for Rev–RRE inhibitors described in publications and patents were based on monitoring the binding of radioislabelled RRE to immobilized Rev [69] or observation of Rev oligomerisation on artificial RRE constructs [261]. A group at GlaxoSmithKline has conducted a high-throughput scintillation proximity assay using surface-bound Rev protein and tritiated RRE RNA to test > 500,000 small molecules for inhibition of Rev–RRE complex formation [69]. Further characterisation of the identified inhibitors revealed, however, that their binding target was the Rev protein and not the RRE RNA.

3.1.2 HIV transactivating region

The HIV TAR RNA domain stimulates transcription of full-length viral genome via binding of the arginine-rich viral Tat protein, which in turn interacts with cellular RNA polymerase II. The observation that a single arginine monomer can bind to TAR RNA with high affinity and thereby block binding of Tar [70] has spurred ongoing efforts to discover other specific small-molecule ligands for the TAR RNA. Among the earliest findings of ligands that bind to TAR RNA and prevent Tat–TAR complex formation were natural aminoglycoside antibiotics [57]. The Tor laboratory at UCSD has used pyrene-labelled TAR RNA to measure binding of various aminoglycosides [71]. They have modified the sugar backbone of a key uridine residue in TAR with a pyrene moiety, the fluorescence of which increases when ligand binding induces a conformational change in the RNA. In a similar approach, the Marino group at the University of Maryland has directly introduced the fluorescent base 2-aminopurine into TAR, which shows fluorescence intensity increase upon ligand binding [72]. A group at Novartis Pharma has reported a fluorescence-based high-throughput assay in which both the Tat–TAR and Rev–RRE target systems were interrogated simultaneously in the same screening mixture [73]. About 110,000 compounds were screened and the resulting hits were analysed for selectivity toward each of the targets as well as potential interference.

The rationale of combining a guanidino group to mimic the TAR–arginine interaction and other RNA-binding scaffolds has led to the investigation of guanidino-substituted trehalose derivatives and β-carbolines by a research group at Peking University [74,75]. The guanidino-trehalose compounds, which resemble the guanidino-aminoglycosides that were extensively studied in the Tor laboratory (see 3.1.1), showed some activity as inhibitors of Tat–TAR interaction in cells [74]. Among the β-carbolines studied, a guanidino-substituted derivative was identified (22, Figure 3) that inhibited Tat-mediated transactivation in cells and reduced virus-related cytopathic effects in HIV-infected cells [75]. Earlier work by the Westhof group in Strasbourg suggested that electrostatic calculations might be applied to predict negatively charged pockets in RNA folds that are potential docking sites for positively charged groups of RNA-binding ligands [76,77]. The method was used to model the binding sites of aminoglycosides in TAR RNA [77]. As an extension of this approach, a group at Ribotargets has conducted an elegant structure-based medicinal chemistry programme on synthetic guanidinium-based Tat–TAR inhibitors [78,79]. A series of disubstituted aryl scaffolds that carried positively charged substituents directed at two specific electrostatic ‘hot spots’ in the TAR RNA fold was designed [78]. The best TAR-binding ligand (23, Figure 3) had an affinity of 1.5 μM for the RNA target, as determined in a proprietary FRET assay that measures dissociation of a Tat–peptide–TAR complex [262]. Subsequent optimisation of the lead compound and replacement of the pharmacologically problematic guanidinium groups yielded a series of amino-bi-aryl heterocycles including an indole derivative (24, Figure 3) with a Kd value of < 100 nM in the Tat–TAR complex dissociation assay [79]. The optimised TAR ligands acted as inhibitors of Tat-induced transcription in vitro at the low micromolar concentration range. Further NMR and biochemical characterisation of ligand binding to the TAR RNA suggest that the bi-aryl heterocycles stabilise an
Figure 3. Structures of ligands for viral RNA targets. 19) 3,8-bis-Urea-ethylenediamine-ethidium and 20) guanidinokanamycin, both by Tor et al. targeting HIV RRE [63,64]; 21) proflavine, discovered as HIV RRE ligand by Marino et al. [8]; 22) guanidino-substituted β-carboline by Yang et al. targeting HIV TAR [75]; 23 and 24) both HIV TAR ligands by Ribotargets [76,77]; 25) acetylpromazine, discovered as HIV TAR ligand by James et al. [81]; 26) 4-(2-pyridyl)-1-piperazinyl-substituted 6-aminquinolone by Palu et al. targeting HIV TAR [87]; 27) ligand for HCV IRES domain IIA and 28) HCV IRES-driven translation inhibitor, both by Isis Pharmaceuticals [268,103]. For discussion, see text.
inactive TAR conformation that is no longer recognised by the Tat protein [79]. This effect, in which a small molecule locks a conformationally flexible RNA domain in a non-functional state rather than directly competes with a binding partner, is comparable to the action of the aminoglycosides on the ribosomal decoding-site. This mechanism has been recognised as a general strategy employed by small-molecule inhibitors directed at RNA targets [2,80].

In an approach that combined in silico screening of ~180,000 commercially available compounds for potential binders of the TAR RNA, the James laboratory at University of California San Francisco has identified a set of 11 ligands that were experimentally confirmed to bind to the RNA target and disrupt Tat–TAR interaction in vitro at micromolar concentrations [81]. The UCSF group used an electrophoretic mobility shift assay to assess RNA binding of the hit compounds that were mostly comprised of flat molecules known to act as intercalators. Among the identified TAR ligands, acetylprazamone (25, Figure 3), a phenothiazine derivative, was considered the most promising lead, as NMR studies revealed that this compound bound specifically to the same TAR site that is recognised by the Tat protein. Determination of the three-dimensional structure of an acetylprazamone–TAR complex by NMR showed that the RNA is locked in a conformation distinct from the Tat–protein-bound TAR [82], suggesting a mechanism of action similar to that observed for the bi-aryl inhibitors described by Ribotargets. Further analysis of the RNA target selectivity of substituted phenothiazines revealed promiscuous binding directed at internal loop structures, including the bacterial decoding site [83]. It is noteworthy that acetylprazamone structurally resembles synthetic aminocarboxylic ligands of TAR that have been developed by a group at Novartis Pharma using a rational design approach [84]. The Novartis aminocarboxylic ligand was also a promiscuous binder of other RNA targets, including the spliceosomal U1A protein-binding loop of U1 snRNA [85].

A promising class of 6-aminquinolone TAR ligands that showed impressive antiviral potency has been discovered by a group at the University of Padova [86-89]. The lead compound, a 4-(2-pyridyl)-1-piperazinyl-substituted 6-aminoquinolone (26, Figure 3), exhibited submicromolar potency (0.6 – 0.9 μM) as an inhibitor of HIV replication in infected cells, and had a K_\text{d} value of 19 nM for TAR RNA binding [87]. The intrinsic fluorescence of the quinolone derivative was exploited for measuring ligand binding to RNA. The compound inhibited Tat–TAR complex formation with an apparent K_\text{d} value of 3.5 μM. Tat-dependent transactivation was reduced by 73% in the presence of 5 μM ligand [88]. Whereas the discovery of the 6-aminquinolone TAR binders was initiated from testing antibacterial quinolone gyrase inhibitors for anti-HIV activity [86], common fluoroquinolone antibiotics such as ciprofloxacin did not show any of the antiviral or RNA binding effects [88]. Initial selectivity testing of the 6-aminquinolone derivatives showed that the compounds target the Tat-binding internal loop of TAR and do not bind to tRNA or DNA [88]. No patent activity has so far resulted from this work.

3.1.3 HIV packaging and dimerisation regions

The packaging region of HIV (Ψ-RNA) is comprised of several RNA stem–loop motifs that are involved in dimerisation and splicing of the viral genome. Dimerisation of HIV mRNA is thought to proceed through a kissing-loop complex that involves the dimerisation initiation site (DIS) within Ψ-RNA. Research at the Institut de Biologie Moleculaire et Cellulaire (IBMC) in Strasbourg has shown that the DIS kissing-loop complex resembles the bacterial decoding-site RNA both on the secondary and tertiary structure levels [90]. As a further similarity between these RNA structures, the DIS complex binds aminoglycoside antibiotics such as paromomycin and neomycin B at a specific single binding site that closely resembles the decoding-site geometry. Footprinting and circular dichroism studies on the whole monomeric Ψ-RNA have shown that aminoglycosides, aminoglycoside dimers and acridine conjugates bind to this RNA target as well [91,92].

3.2 RNA targets of the hepatitis C virus

The RNA genome of hepatitis C virus (HCV) has attracted drug discovery efforts and targeting conserved RNA elements that reside in the 5’- and 3’-noncoding regions and are involved in viral replication. In particular, the internal ribosome entry site (IRES), a highly structured region of several-100 nucleotides within the 5’-terminus of the HCV RNA genome, has been the subject of several drug discovery programmes. The extensive coverage of intellectual property around HCV and its genome by Chiron Corp., where the virus was discovered in 1987, creates a peculiar environment for commercial research on HCV [93]. Earlier work on the HCV IRES target has been compiled in some comprehensive reviews [58,94,95]. Recent developments in this area are focused upon here.

The HCV IRES is comprised of several structured subdomains that are connected by flexible stretches of single-stranded RNA. Upon association with host cell 40S ribosomal subunits the HCV IRES RNA adopts a well-defined overall fold [96] that induces assembly of functional ribosomes and translation initiation at the viral mRNA. Academic and industrial laboratories have used NMR and X-ray crystallography to determine the three-dimensional structures of HCV IRES subdomains [97-101]. The related publications emphasise potential exploitation of the different subdomains as drug targets. Consequently, patent applications that describe the whole HCV IRES, its subdomains, and host cell complexes thereof as potential targets for the development of antivirals have been filed [263-267]. The HCV IRES subdomain IIIe has been used as a target for screening 300,000 compounds in an MS-based high-throughput screen at Eli Lilly and Company [102]. A total of 11 compounds showed activity as inhibitors of IRES-dependent
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translation and were confirmed as ligands with dissociation constants in the micromolar to millimolar range. Three of the ligands that had $K_d$ value of < 10 µM for the IRES IIIe subdomain were aminoglycosides. The identity of the aminoglycosides was not disclosed, and neither was the inhibition selectivity for IRES-dependent translation versus the translation of cellular mRNAs.

In a similar MS affinity screening campaign against subdomain IIa, Isis Pharmaceuticals discovered benzimidazoles as ligands of the IRES RNA, and disclosed them in a recent patent application [208]. The most potent compounds (27, Figure 3) had a better than 1 µM affinity for the IIa RNA and displayed activity as inhibitors of IRES-dependent translation with IC$_{50}$ values in the low micromolar range. The selectivity of the benzimidazoles for the IRES target versus translation of cellular mRNAs has not been reported. Initial acute in vivo toxicity and single-dose pharmacokinetic studies revealed no significant toxicity at doses up to 45 mg/kg in mice and satisfying distribution in target tissues after oral administration. Isis Pharmaceuticals has also applied for patent coverage of biaryl guanidine derivatives as inhibitors of HCV IRES-dependent translation; however, without disclosing the specific target site on the RNA [209]. The lead structure was discovered in a high-throughput transcription/translation screen of ~ 180,000 compounds [103]. The most potent compounds inhibited viral translation with IC$_{50}$ values of 2 – 3 µM (28, Figure 3). Selectivity of the guanidines for the IRES-dependent process was moderate (~ 2.5-fold for the most potent translation inhibitors) [103].

4. Expert opinion

The patent literature covering the development of RNA-targeted small-molecule therapeutics reveals an intriguing asymmetry: patent distribution is clustered around the extremes (the very early and late stages) of the drug discovery process. Target definition and screening methodologies are the focus of many applications related to initial explorations. On the other hand, composition of matter claims surrounding established chemical classes of RNA-directed ligands represent the outcome of drug development efforts. These often result from mature discovery programmes that were driven in the absence of explicit knowledge of the RNA targets. In between these extremes, basic research on model ligands is found, predominantly in academic laboratories, whose primary goal is the fundamental exploration of RNA recognition and, to a lesser extent, the discovery of novel RNA-directed ligands as leads for drug development.

Two examples, the ribosome-directed antibiotics and the recently discovered TAR-binding aminoquinolone derivatives, reflect the impact of increasing knowledge of RNA molecular recognition on drug discovery. Many decades span the initial discovery of most of the ribosome-directed antibiotics and the experimental proof demonstrating that these compounds target the ribosomal RNA [6]. Even for the most recently emerging class of antibiotics, the synthetic oxazolidinones, which were discovered well into the age of advanced molecular biology, knowledge of their RNA binding site and three-dimensional structure is only beginning to be exploited for drug development [37]. In contrast, only a few years passed between the discovery of the antiviral aminoquinolones as in vivo active compounds and the realisation that they target the TAR RNA [89]. A budding notion of RNA targets in HIV, at the time when the antiviral activity of the aminoquinolones was first described, certainly facilitated subsequent research efforts towards uncovering TAR as the molecular target. With the rapid discovery of the aminoquinolone–TAR interaction in mind, it will be exciting to observe how knowledge of RNA–ligand recognition, and perhaps structural data, will be used to advance these novel antivirals.

Just as the use of structural data are now an integral part of drug discovery [104], the concept of RNA as a drug target is rapidly establishing itself both in academic and industrial research. Early successes that start filling the discovery gap with new chemical classes of RNA ligands have been reported. Whereas good progress has been made towards increasing the affinity of RNA-directed compounds, target selectivity and specificity remain the single most important challenge to be addressed by these drug discovery efforts. Examples such as the oxazolidinones and aminoquinolones, which comprise structurally simple, drug-like scaffolds, will lead the way to the discovery of novel specific ligands for RNA targets. In parallel, as RNA continues to reveal the extent of its involvement in cellular functions, additional novel targets of therapeutic potential will emerge.

Bibliography

Papers of special note have been highlighted as either of interest (*) or of considerable interest (**) to readers.

• Up-to-date overview on the chemical diversity of natural small-molecule ligands for RNA, focusing on bacterial ribosomal RNA targets and related 3D structural data.


• First published crystal structure of a designed aminoglycoside derivative in complex with its RNA target. The conception, synthesis and identification of the compound is outlined in [12].


• Use of crystal structure data for the design of an aminoglycoside mimetic in which the 2'-deoxystreptamine scaffold of the natural antibiotics was replaced by a heterocyclic system aimed at utilizing a target-bound water molecule.


• Extension of a mass spectrometry-based affinity assay for RNA targets to establish SAR and to guide lead improvement (SAR by MS).

• First publication of an NMR-based screening approach for an RNA target, yielding a series of novel scaffolds for molecular recognition of the bacterial decoding site.


• Comprehensive description of a lead discovery campaign for the bacterial decoding site, including computational screening, biochemical assaying and NMR-based characterisation of compounds. Data are provided for > 30 hits out of the discovery effort.


• Comprehensive outline of fluorescence-based techniques for the discovery and systematic evaluation of several Rev–RRE inhibitor classes. Emphasis is given to assess both the affinity and specificity of the RNA ligands.


• Systematic study of the relationship between target binding affinity and specificity of RNA-directed ligands, using the HIV Rev–RRE complex as a model system.


- This paper and [78] describe comprehensively a structure-based medicinal chemistry programme focusing on the TAR RNA target. The stepwise procedure from the initial compound design to iterative rounds of ligand improvement is outlined along with NMR structural studies on the ligand–RNA complexes.


**This paper and [87] describe a novel class of Tat–TAR inhibitors based on 6-aminoquinolones, which were originally discovered by compound screening against infected cell lines. Investigation of the mechanism of action and molecular target of the novel inhibitors is outlined. The publications describe an approach that is exemplary and unique in the field of targeting viral RNA as they outline an inverse discovery route, that is, tracing a path from an empirically observed *in vivo* activity to a molecular RNA target–ligand interaction.**


RNA as a target for small-molecule therapeutics


Patents


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