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Recent Advances in Developing Small Molecules Targeting RNA

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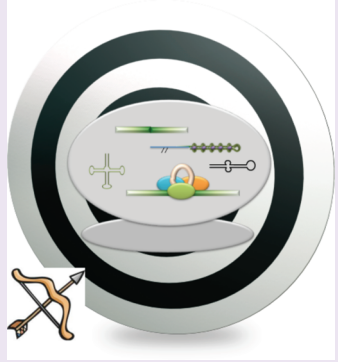
ABSTRACT: RNAs are underexploited targets for small molecule drugs or chemical probes of function. This may be due, in part, to a fundamental lack of understanding of the types of small molecules that bind RNA specifically and the types of RNA motifs that specifically bind small molecules. In this review, we describe recent advances in the development and design of small molecules that bind to RNA and modulate function that aim to fill this void.



Ribonucleic acid (RNA) is an essential macromolecule that has diverse functions *in vivo*. For example, RNA regulates transcription and translation,[1,2] catalyzes protein synthesis,[3] and controls gene expression.[4,5] Many of these functions have been uncovered in the past decade, increasing the number of potential RNA targets for small molecule chemical genetics probes or therapeutics. Despite these discoveries, there are comparatively few compounds that target RNA with high affinity and specificity. This is in contrast to the large number of small molecules that target and modulate the biological function of DNA and protein.[6,7]

Although there are nearly 100 naturally occurring modified nucleotides in RNA,[8,9] it is transcribed from only four nucleobases. In this regard, RNA is chemically similar to DNA and less complex than protein. Despite their chemical similarity, there are many more known classes of small molecules that bind DNA. This is mainly due to the number of bioactive natural products that bind to and target DNA through base stacking (intercalation) and by interacting with the minor groove.[10] The minor groove in DNA helical structure is a well-exploited target for small molecules. For example, custom assembled polyamides can be used to sequence-specifically read out hydrogen bond donors and acceptors in the minor groove.[11] There are far fewer examples of small molecules that target RNA grooves. This is likely due to the fact that the major groove of an A-form RNA helix is deeper and narrower than the major groove in B-form DNA, while the minor groove is more shallow.

The 3-dimensional structures that DNA and RNA adopt *in vivo* are very different. Whereas DNA is typically helical due to being double stranded, RNA folds into diverse structures, adopting folds that are more similar to proteins. This is because RNA is single stranded and folds onto itself to minimize its energy. RNA structure often has unique binding pockets for small molecules, and its structural diversity could be exploited to design small molecules that specifically target an RNA of interest. To date, the most well described binding of small molecules to a highly structured RNA is the binding of antibiotics to the ribosome.[12]

Because RNA's structural diversity should provide the potential for selective recognition by small molecules, targeting RNA could be a strategy for treating diseases. There is a dearth of information, however, about the chemical scaffolds that are privileged to bind RNA. The end result is that high-throughput screening campaigns for RNA targets are far less successful than protein targets because small molecules libraries employed in these efforts are biased for binding proteins.

The most well documented classes of compounds that modulate RNA function include antibiotics such as aminoglycosides, macrolides, tetracyclines, and oxazolidinones. In general, these compounds and derivatives thereof have modest affinities and selectivities for RNA. Efforts have been made to use these privileged scaffolds as building blocks to target RNA secondary structures, such as bulges[13,14] and helices.[15] This review summarizes recent progress made to address the RNA-targeting problem since the very thorough review by Thomas and Hergenrother was published.[16] Specifically, we discuss small molecules that target bacterial RNAs, viral RNAs, and messenger (m)RNAs, and the methods that were used to design them will be discussed.

SMALL MOLECULES THAT TARGET THE RIBOSOME

Of all validated RNA targets, the ribosome is by far the most thoroughly studied. The vast majority of antibacterials exert their activity by modulating ribosome function, including the

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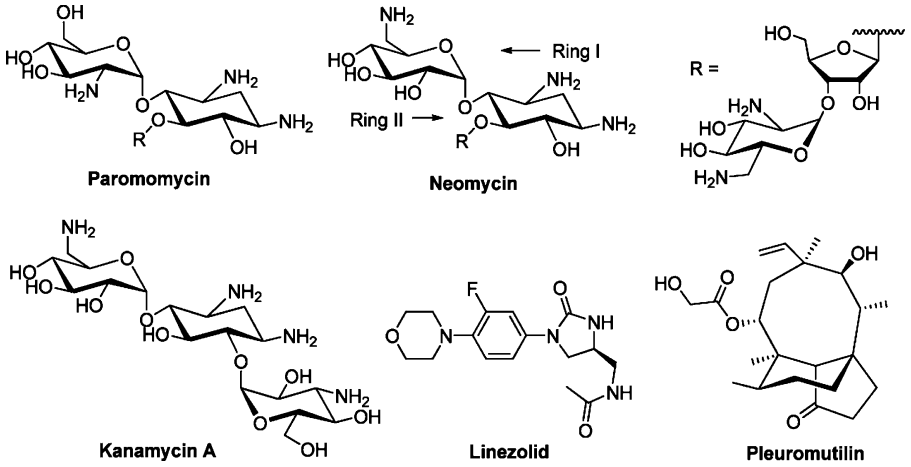

Figure 1. Chemical structures of antibiotics that bind to the 50S or 30S subunit of the bacterial ribosome, including aminoglycosides (neomycin, paromomycin, and kanamycin A) and oxazolidinones (linezolid and pleuromutilin).

ribosome's ability to discriminate between cognate and noncognate tRNAs. The ribosome is an ideal target for antibacterials because of its essential function in protein synthesis and its high abundance relative to other RNAs.[17−23] The prokaryotic ribosome contains two subunits, 30S (small) and 50S (large), which are composed of 65% ribosomal RNA (rRNA). The small subunit is responsible for proofreading, ensuring that the correct tRNA is loaded in the aminoacyl tRNA (A)-site before polypeptide synthesis continues. The large subunit catalyzes polypeptide bond formation. A variety of antibiotic classes, such as aminoglycosides, oxazolidinones, and pleuromutilin, have been identified that bind to defined regions of the ribosome, resulting in the perturbation of protein synthesis (Figure 1).[12,16,24]

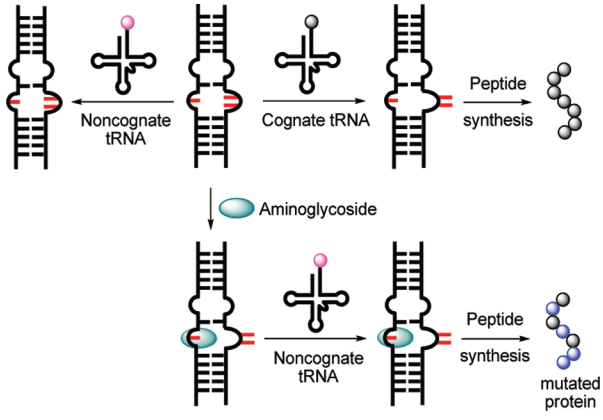

Figure 2. The 16S A-site proofreads aminoacyl-tRNAs. Elongation of the polypeptide occurs when a cognate tRNA binds in the A-site inducing an extrahelical conformation (A1492 and A1493 are flipped out of the helix). Aminoglycosides bind to the A-site and induce the same extrahelical conformation, resulting in the mis-incorporation of amino acids into the polypeptide.[25]

In the small 30S subunit, the 16S rRNA forms the A-site and binds tRNAs. If a cognate tRNA is present, two adenine residues (A1492 and A1493) are flipped out of the helix (opened form), resulting in the incorporation of the correct amino acid into the polypeptide (Figure 2).[25] To date, the A-site is the most explored RNA drug target mainly due to its direct role in ensuring fidelity in the translation of the genetic code. Aminoglycoside antibiotics are highly positively charged compounds that bind the A-site and force the A-site into the open form, allowing recognition of noncognate tRNA that results in amino acid mis-incorporation (Figure 2). Aminoglycosides are broad-spectrum antibiotics that have been widely used since streptomycin was first discovered in 1940s. Many bacterial strains, however, have evolved resistance to this class of antibiotics mainly *via* enzymatic modification resulting in reduced affinity of the small molecule for the ribosome.[26]

Many groups have developed new antibiotics based on the aminoglycoside scaffold. These derivatives have increased affinities for the A-site and/or reduced affinity for aminoglycoside-modifying enzymes.[16] Recently, Vourloumis and coworkers developed spiro-compouds (**1**−**3**, Figure 3) utilizing the 2-deoxystreptamine (2-DOS) scaffold (Figure 3), the core building block (ring II) of neomycin and paromomycin (Figure 1).[27] Using a fluorescence-based assay, the half-maximal response concentration (EC_{50} values) of these compounds for binding to the A-site were determined to be in the micromolar range. Binding affinity was improved by introducing a hydrophilic side chain to 2-DOS. More potent 2-DOS derivatives (spiro-ethers) were synthesized and evaluated by the same group,[28] all of which contained a triazole ring with various substituents. Eight of the 12 designed compounds (examples **4** and **5**, Figure 3) have nanomolar EC_{50} values for binding to the bacterial 16S A-site RNA. The nature of the substituents had little effect on affinity provided it was not overly bulky, suggesting that the triazole ring itself was responsible for high affinity binding. It was proposed that the triazole ring either forms a hydrogen bond with N7 of A1493 or interacts with the phosphate backbone of G1494.

Compared to other RNA drug targets, there is a wealth of structural information describing the binding of small molecules to the ribosome. This information has been used to design improved compounds, as was the case for oxazolidinone analogues.[29] During protein synthesis, peptide bond formation occurs at the peptidyl transferase center (PTC). Many antibiotics, such as linezolid and pleuromutilin (Figure 1), bind to the PTC region of the 50S subunit and interrupt peptide formation or perturb protein elongation. Linezolid, an oxazolidinone antibiotic, was approved for clinical use in 2000. Recently, the crystal structure of linezolid bound to the 50S A-site, near the PTC, was published.[30] Using this structural information of the juxtaposition of the linezolid and

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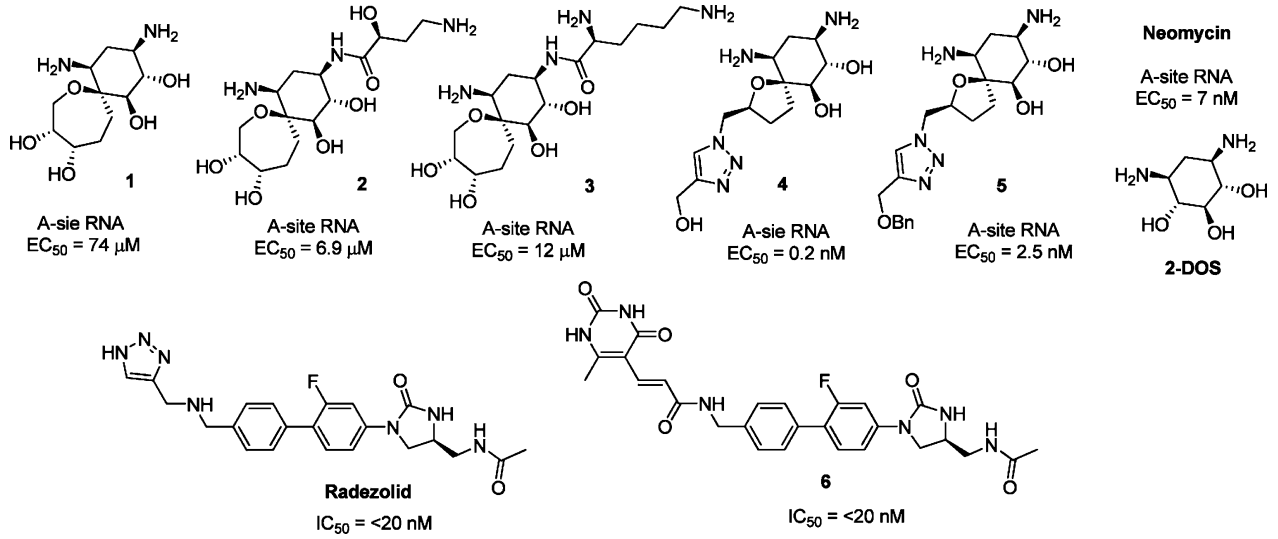

Figure 3. Recently developed small molecules that bind to the ribosomal A-site.[27−29]

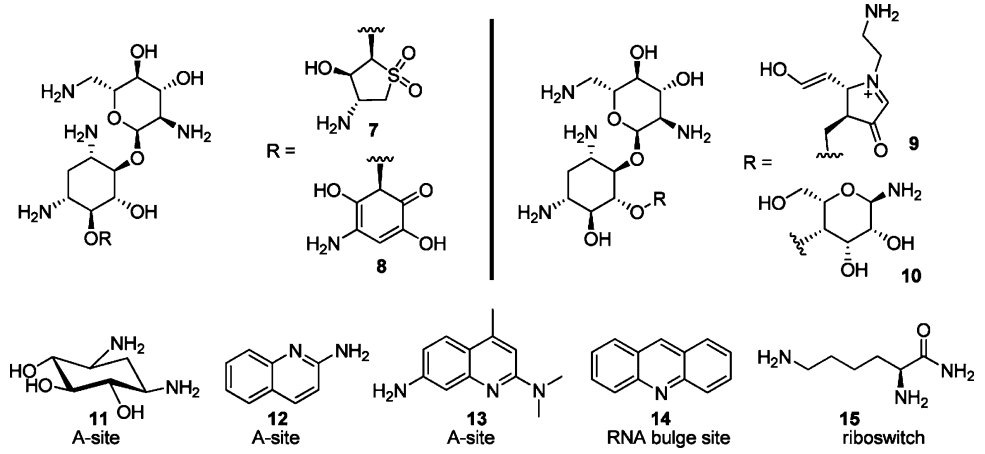

Figure 4. Potential ligands that bind the ribosomal 16S A-site identified by virtual screening and RNA-directed fragment screening.[31,32]

sparsomycin binding sites in the ribosome, Franceschi and co-workers at Rib-X developed a series of oxazolidinone analogues.[29] These compounds were designed to link two antibiotics by an optimal bridging element, which improves the interactions and shape complementarity with the ribosome.[29] The compounds bind to the 50S A-site and inhibit translation in linezolid-resistant *Staphylococcus aureus* in the nanomolar range (Figure 3). The analogues increase translational inaccuracy compared to linezolid by promoting nonsense suppression and frameshifting.[29] One of the compounds, radezolid (Figure 3), is currently in clinical trials.

Other groups have employed fragment-based screening to design aminoglycoside derivatives with improved properties. Trylska and co-workers recently combined fragment-based virtual screening and 3D quantitative structure activity relationship (3D-QSAR) scoring to search for new A-site binding scaffolds based on the neamine core of neomycin (ring I and ring II; Figure 1).[31] The suitable fragments in a 90,444-member library were obtained by screening the compounds against two pharmacophores designed using the crystal structure of paromomycin bound to 16S RNA. The fragments were assembled onto the neamine core and scored with the 3D-QSAR model using known biological data of aminoglycosides. Four ligands (**7**−**10**) were predicted as potent antibiotics (Figure 4).[31] Aboul-ela and co-workers developed a RNA-directed fragment screening approach in which a library containing 102 RNA-focused fragments was created based on known RNA binding ligands.[32] The lead compounds were identified by clustering the fragment library followed by screening *via* waterLOGSY NMR spectroscopy. Five compounds (**11**−**15**) bind to the 16S A-site, but two of them also likely bind to RNA bulges or riboswitches (Figure 4).

By examining both the structure of the RNA binding pocket and the interactions between the RNA and the small molecules, generalities can be made in order to aid the design of improved compounds. These generalities have been used to define scoring functions such that computational screening can be completed as has been previously reported for proteins.[33] One such computational tool, RiboDock,[34] uses available information from structural studies to search for new scaffolds that target an RNA of interest.

TARGETING VIRAL RNAS

Retroviruses, including human immunodeficiency virus type 1 (HIV-1) and hepatitis C virus (HCV), have RNA genomes that are reverse transcribed and incorporated into the host's DNA upon infection. The host's cellular machinery is then used to produce copies of the virus. Small molecules that specifically

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target the virus's RNA structure and inhibit replication could serve as viable therapeutics for treating viral infections.

Targeting HIV RNA. After the HIV genomic RNA is integrated in the host cell's genome, transcription of viral DNA is facilitated by HIV trans-activator of transcription (Tat) protein. Tat recognizes and binds to a bulged RNA hairpin loop called trans-activating response (TAR) element, which is located at the beginning of viral transcripts. The binding of cyclin T1 (an endogenous host protein) with the Tat-TAR complex further enhances viral RNA transcription. Disruption of the Tat-TAR complex, thus inhibiting viral replication, is considered a promising alternative antiviral approach to inhibiting viral proteins such as integrase and reverse transcriptase.

The aminoglycoside class of antibiotics inhibits the Tat-TAR complex. Neomycin (Figure 1) is the most potent inhibitor with an IC_{50} of 0.92 μM.[35] On this basis, Arya and co-workers synthesized a series of neomycin dimers (**16**–**18**) with different linkers (Figure 5).[36] The triazole-linked neomycin dimers

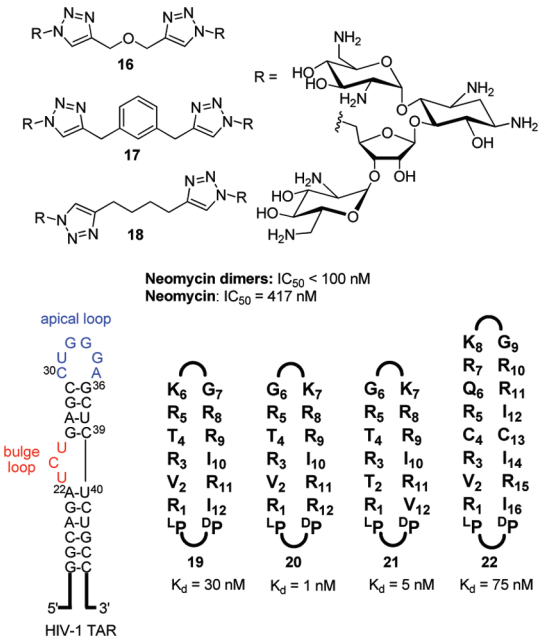

Figure 5. Examples of molecules that target HIV-1 TAR RNA.[36,37,39]

displaced ethidium bromide from an HIV-1 TAR RNA construct with an IC_{50} of less than 100 nM. It was suggested that the binding affinity of neomycin for TAR RNA was improved by one neomycin binding to the lower stem region and the other binding to the upper stem or hairpin region.

Peptidomimetics also inhibit the Tat-TAR complex. While linear polypeptides bind to RNA nonspecifically due to their high flexibility, Varani and co-workers developed conformationally constrained cyclic peptide mimics of Tat that bind to HIV-1 TAR with high specificity and affinity (Figure 5).[37] Among 100 peptides screened, three arginine-rich peptidomimetics (**19**–**21**) bind tightly to TAR with low nanomolar dissociation constants. The proteolytically stable cyclic peptides are active against a wide range of viral isolates and are not cytotoxic to host cells when dosed up to 1 mM. Structural analysis suggests that the cyclic peptides adopt a rigid β-hairpin structure imposed by the D-Pro-L-Pro template, with the side chains of peptides emerging from each face. The interaction between Arg-11 in the peptidomimetic and the highly conserved A35 of TAR forces the bulge and loop region close to the peptide, creating a deep pocket that Tat binds with much weaker affinity.[37] Additional studies revealed that cyclic peptidomimics of Tat also induce unique dynamic changes in the apical loop, potentially interfering with cyclin T1 binding.[38] By screening a library of extended cyclic peptide mimics of Tat, it was determined that three amino acid residues (Arg7, Lys8, and Ile12 in **22**) are critical for high affinity and specific TAR binding (Figure 5).[39]

A virtual screening approach was also employed to identify small molecules that disrupt the Tat-TAR complex. Using molecular dynamics (MD) and NMR residual dipolar coupling (RDC), Al-Hashimi and co-workers showed that HIV-1 TAR RNA folds into a dynamic ensemble of structures.[40,41] They then used the Internal Coordinate Mechanics (ICM) docking program to identify small molecules in a 51,000-member library that potentially bind to the RNA without changing its conformation.[40,41] Six of the top 57 hits from the screen, including aminoglycosides, mitoxantrone, and 5-(*N*,*N*)-dimethylamiloride (DMA) (Figure 6), bind TAR with high affinity

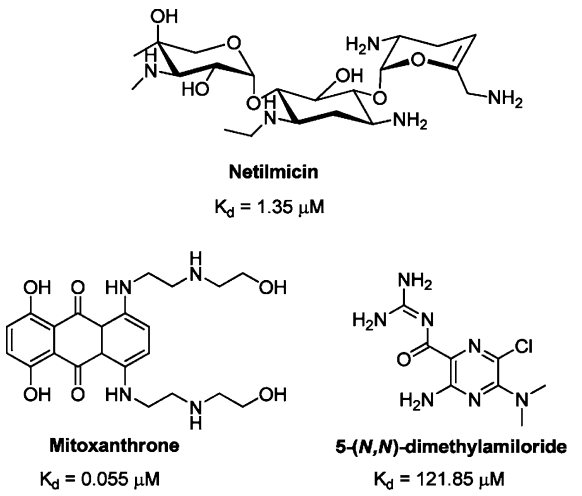

Figure 6. Small molecules identified from virtual screening that bind HIV-1 TAR.[40,41]

as determined from fluorescence-based assays. Among them, netilmicin (an aminoglycoside) inhibits replication in an HIV-1 indicator cell line (TZM-bl) and the HIV-1 NL4-3 isolate with an IC_{50} value of ~23.1 μM.[41] NMR chemical shift mapping studies were used to gain insight into the binding modes of the different small molecules classes: aminoglycosides bind to the bulge, upper stem, and apical loop of TAR; mitoxantrone (Figure 6) forms a unique stack on G26 in the upper stem; and DMA (Figure 6) binds to a unique pocket within the TAR apical loop.

The translation of HIV-1 *pol* gene requires a −1 ribosomal frameshift between the *gag* and *pol* reading frames to produce the proper Gag-Pol fusion protein.[42] The frameshift event occurs with 5−10% efficiency and is induced by two highly conserved RNA elements, a heptanucleotide slippery sequence (UUUUUUA) and a downstream stem-loop structure (Figure 7).[42,43] The stem-loop directly regulates frameshift efficiency, and altering its structure or stability by small molecules may inhibit HIV-1 replication.[44] Butcher and co-workers reported that the highly positively charged guanidinoneomycin B (Figure 7) binds to the stem of the downstream step-loop structure.

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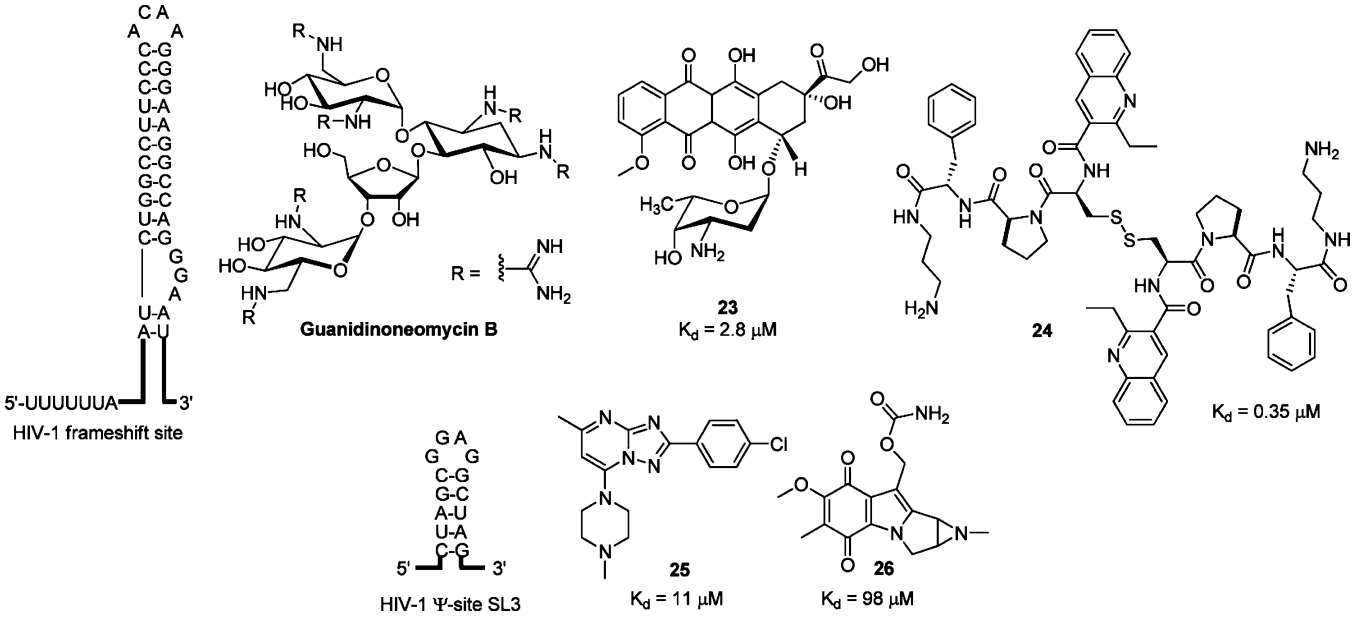

Figure 7. Examples of small molecules that bind to the HIV-1 frameshift stem-loop structure and SL3 of the HIV-1 Ψ-site.[45,46,48]

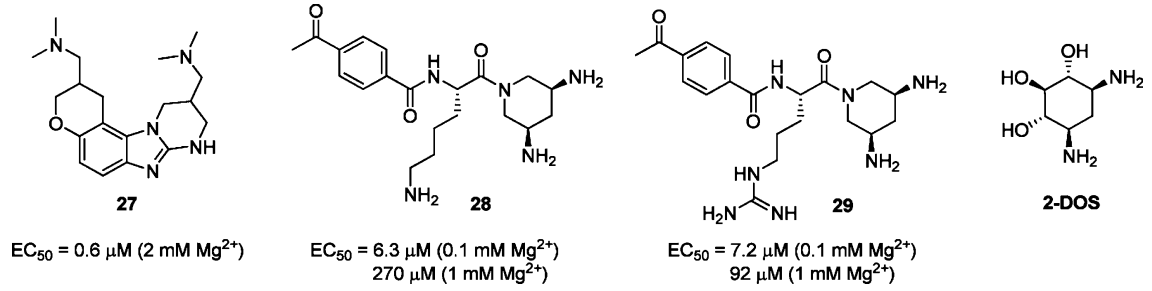

Figure 8. Small molecules that bind to the hepatitis C virus internal ribosome entry site (IRES) RNA.[52−54]

Specifically, it binds within the electronegative major groove of a CG pair and increases the thermodynamic stability of the stem-loop.[45] Although the specificity and affinity of this molecule needs to be further studied, the guanidinoneomycin B scaffold could be utilized for developing new compounds that bind the frameshift element. Later on, the same group screened a 34,500-compound library and identified doxorubicin (**23**), a cancer chemotherapy drug, that binds to the HIV-1 frameshift site with low micromolar affinity (Figure 7). It was suggested that **23** intercalates into the GGA bulge region and stabilizes the lower helix of RNA, resulting in the decrease of translational frameshift efficiency by 28% *in vitro*.

A disulfide tetrapeptide dimer (**24**, Figure 7) and its carba analogues (the disulfide is replaced with an olefin or hydrocarbon) were synthesized and evaluated for binding to the HIV-1 frameshift stem-loop by Miller and co-workers.[46] The dimers bind to the target RNA with nanomolar K_d values. The 2-ethyl-3-carboxyquinoline moieties and peptide residues are most likely the primary source of affinity and selectivity, respectively.

Other HIV-1 RNA structures, such as the packaging element Ψ (Ψ-site), are also the targets for the small molecules that inhibit viral replication. The Ψ-site is composed of four stem-loops, SL1−SL4, in which SL3 is a highly conserved GGAG hairpin (Figure 7). The hairpin structure is critical for efficient encapsidation of HIV-1 genome.[47] Baranger and co-workers used computational screening and tools to identify compounds from the NCI library that specifically bind to SL3. Nine lead compounds were identified, two of which bind to SL3 hairpin with affinities in micromolar range (**25**, **26**; Figure 7).[48]

Targeting HCV. The internal ribosome entry site (IRES) of HCV binds to the host cell's ribosomal 40S subunit and initiates viral translation in a cap-independent fashion.[49] The crystal structure of the IRES subdomain IIa, reported by Hermann and co-workers, showed that it adopts an L-shaped conformation stabilized by divalent metal ions.[50] It is likely that maintaining this architecture is critical to IRES-40S complex formation.[51] Seth and co-workers identified a benzimidazole hit with a K_d of ∼100 μM to an RNA model of IRES IIa from a 180,000-member library using mass spectrometry-based high-throughput screening method.[52] A new class of benzimidazoles, including **27** (Figure 8), were subsequently developed and evaluated with submicromolar affinity to the IIa RNA construct.[52] Hermann and co-workers used a FRET assay to identify that **27** binds to an oligonucleotide mimic of IRES IIa with an EC_{50} value of 0.6 μM in 2 mM Mg^{2+}.[52,53] In addition, **27** inhibits HCV replication with EC_{50} of 5.4 μM, most likely by changing the angle of the L-shape architecture.[53] In contrast, 2-DOS derivatives **28** and **29** (Figure 8) arrest the L-shape IIa RNA in the bent state by competing with Mg^{2+} and thus are likely to inhibit the transition of the HCV translation complex.[54]

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TARGETING RIBOSWITCHES

Riboswitches, usually located in 5′ untranslated regions (UTRs) of mRNAs, are composed of two functionally distinct domains: an aptamer domain and an expression platform. The aptamer domain binds to a ligand, inducing a structural change in the expression platform. The structure of the expression platform regulates the expression level of the adjacent open reading frame (ORF). To date, at least 20 distinct classes of riboswitches have been reported that are recognized by different small molecule metabolites, divalent cations, or second messengers.[55−58] Since riboswitches have evolved as small molecule receptors and are found almost exclusively in bacteria, they are ideal targets for antibiotics. Several small molecule analogues targeting the thiame pyrophosphate (TPP, Figure 9),[59] flavin mononucleotide (FMN),[60] lysine,[61] T-box

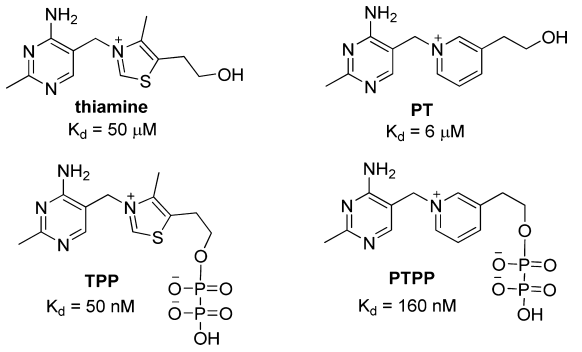

Figure 9. The structures of thiamine and its analogues that bind the *B. subtilis tenA* TPP riboswitch.[59,67−69]

(monitors aminoacylation of tRNA),[62,63] and purine riboswitches[64,65] have been rationally designed based on atomic-resolution structures of the target RNA bound to the corresponding response-inducing metabolites.[66]

Although most riboswitches are found only in bacteria, the TPP riboswitch is found in both bacteria and eukaryotes. TPP (Figure 9) is the active form of intracellular thiamine, acting as an essential coenzyme for the catalytic cleavage of a carbon−carbon bond in many biochemical reactions.[67] An analogue of thiamine, pyrithiamine (PT, Figure 9), has been shown to be toxic to fungi and bacteria.[68,69] Analogous to TPP, the active form of PT, pyrophosphate pyrithiamine (PTPP, Figure 9) is produced from the phosphorylation of PT in the cell. PTPP binds to the TPP riboswitch with high affinity[59] and reduces the expression of a downstream reporter gene by 5-fold. It was proposed that the binding of PTPP to the TPP riboswitch represses the expression of genes associated with TPP transport and biosynthesis and thus inhibits cell growth.[59] PT-resistance was observed due to a mutation in the TPP riboswitch, which disrupted binding of PTPP; TPP transport and biosynthesis genes were expressed and cell functionalities were restored.[59]

FMN riboswitches are located in the 5′ UTR of prokaryotic mRNAs that encode for FMN (Figure 10) transport and biosynthesis proteins. Roseoflavin (Figure 10), a pigment from *Streptomyces davawensis*, is an analogue of riboflavin and FMN and is an antimicrobial. It binds directly to the FMN riboswitch aptamer domains with high affinity and down-regulates expression of an FMN riboswitch-*lacZ* reporter gene in *Bacillus subtilis* by 5-fold.[60] Interestingly, mutations within the FMN riboswitch that emerged in roseoflavin-resistant bacteria

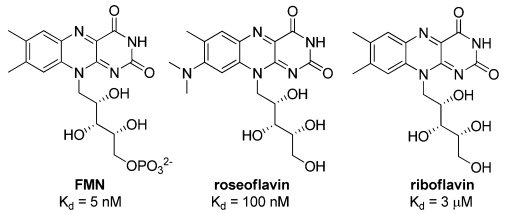

Figure 10. The structures of FMN and its analogues that bind the *B. subtilis tenA* FMN riboswitch.[60]

disrupted roseoflavin binding, a reduced regulatory response was observed.

Several L-lysine analogues have been identified that bind to the lysine riboswitch with low micromolar affinities (Figure

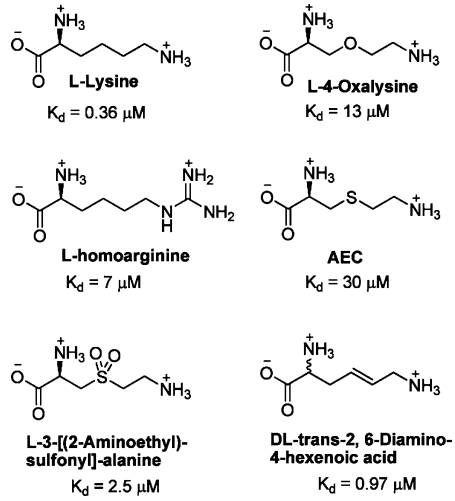

Figure 11. The structures of L-lysine and its analogues that bind the *B. subtilis* lysine riboswitch.[70,71]

11).[70] Among the 12 analogues that were evaluated, L-4-oxalysine L-3-[(2-aminoethyl)-sulfonyl]-alanine and DL-*trans*-2,6-diamino-4-hexenoic acid (Figure 11) inhibit *B. subtilis* growth at least partially *via* a riboswitch-dependent mechanism.[70] Although L-aminoethylcysteine (AEC, Figure 11) binds to the lysine riboswitch with moderate affinity (K_d = 30 μM) and exhibits antibacterial activity, a riboswitch-independent mechanism may be involved. It has been suggested that lysyl-tRNA synthetase (LysRS) is the primary cellular target of AEC, which binds with similar affinity to L-lysine (K_d = 1.7 μM).[71]

The T-box riboswitch responds to uncharged cognate tRNAs and regulates transcription by changing the structure of the antiterminator RNA element.[72] A small library of oxazolidinones has been screened for disrupting a T-box riboswitch. Two compounds were identified that have low micromolar K_d values (**30**, **31**; Figure 12) and modulate antitermination activity *in vitro*.[62,63] Interestingly, **30** led to reduced tRNA-dependent antitermination, and **31** led to enhanced tRNA-independent antitermination, suggesting that the two compounds have different binding modes.[63]

Purine riboswitches recognize a broad range of purines, including guanine,[73] adenine,[73] deoxyguanosine,[74] and hypoxanthine[75] (Figure 13). Batey and co-workers evaluated several purine and pyrimidine analogues and identified that 2,6-diaminopurine and 2,4,6-triaminopyrimidine bind to purine riboswitches with high affinity. These data suggest that

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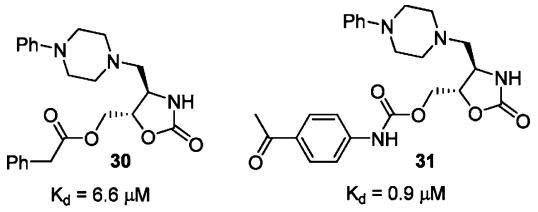


Figure 12. The structures of oxazolidinones that bind T-box riboswitches.[62,63]

modification at the 5- and/or 6-postions of the pyrimidine ring imparts specific binding (Figure 13).[76] Breaker and co-workers screened 16 guanine analogues with modifications at the C2 or C6 position. Even though all 16 compounds bind the riboswitch with high affinity (dissociation constants ranging from 0.5 nM to 3.3 μM), only 2-amino-N^6-hydroxyadenine (Figure 13) repressed expression of a guanine riboswitch-regulated reporter gene. Some of the compounds inhibited bacterial cell growth, suggesting different modes of action.[64] Another pyrimidine derivative, 2,5,6-triamino-pyrimidine-4-one (Figure 13), inhibits the bacterial growth in a mouse model, likely by inhibiting expression of proteins regulated by guanine riboswitches.[77]

Methods have been developed to screen libraries of small molecules that bind to riboswitches and modulate their functions.[78−80] A virtual screening approach that employed the program DOCK[81−84] was used to facilitate the screening of small molecules that target riboswitches.[80] Docking calculations were modified using structural information about purine riboswitch-ligand complexes. Four compounds were identified from the screen with binding affinities ranging from 80 to 650 μM.[80]

A fragment-based screening approach in conjunction with biophysical techniques (such as equilibrium dialysis, water-LOGSY and T2 relaxation-edited NMR spectroscopy, and isothermal titration calorimetry) has also been developed.[78,79] Competition dialysis was used to screen a fragment library of ~1300 small molecules against the TPP riboswitch using radiolabeled thiamine. Binding of the 17 hits from competition dialysis was further investigated by waterLOGSY NMR spectroscopy and ITC. The compounds bind with K_d's ranging from 22 to 670 μM. Although these compounds failed to regulate gene expression in an *in vitro* translation assay, this promising approach may be used to screen and identify more potent molecules from larger libraries.

TARGETING EXPANDED NUCLEOTIDE REPEATS: MYOTONIC DYSTROPHY

Nucleotide repeat expansions cause a number of diseases, including Fragile X Syndrome (FXS), Fragile X-associated Tremor/Ataxia Syndrome (FXTAS),[85] myotonic dystrophy type I (DM1)[86] and type II (DM2),[87] Huntington's disease (HD),[88] and numerous spinocerebellar ataxias (SCAs).[89,90] These disorders all result from different expanded nucleotide repeats located in different regions of a transcript (untranslated regions, coding regions, and introns) (Figure 14). Notable among these targets are the RNAs that cause the myotonic dystrophies, incurable forms of muscular dystrophy.

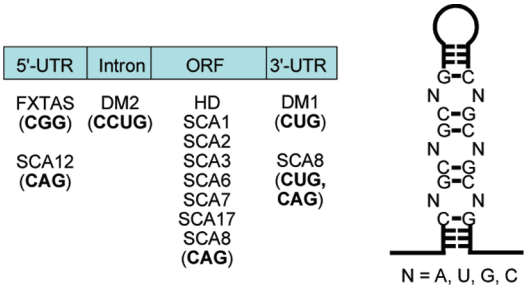
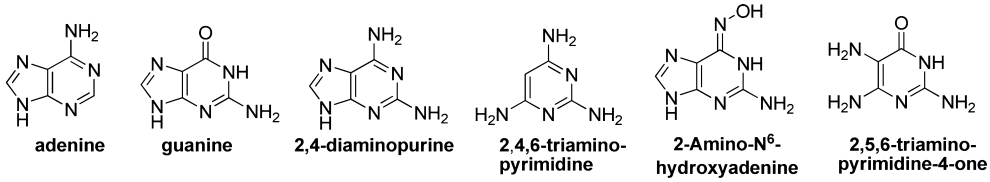


Figure 14. Expanded nucleotide repeats are located in different regions of RNA transcripts. All lead to disease.[85−90]

Myotonic dystrophy (DM) is an autosomal dominant genetic disease caused by either a trinucleotide repeat (CTG) in the 3′ UTR of the dystrophia myotonica protein kinase (*DMPK*) gene (DM1)[91] or a tetranucleotide repeat (CCTG) in intron 1 of the zinc finger 9 protein (*ZNF9*) gene (DM2).[92] DM1 and DM2 have the same disease pathologies: the expanded repeats fold into a hairpin displaying regularly repeating internal loops that are high affinity binding sites for the splicing regulator, muscleblind-like 1 protein (MBNL1).[93−97] Sequestration of MBNL1 causes the disregulation of the alternative splicing of various pre-mRNAs including cardiac troponin T (*cTNT*), the insulin receptor (*IR*), and sarcoendoplasmic reticulum Ca^{2+} ATPase 1 (*SERCA1*), among others.[86,95,98−103] There are other defects associated with DM, including a translational defect of the *DMPK* mRNA[103,104] and the formation of nuclear foci.[93,94,105−110]

The disease mechanism for expanded r(CUG) and r(CCUG) repeats points to a therapeutic strategy: a high affinity small molecule could displace proteins from the expanded repeats and restore the proper function of MBNL1 (Figure 15). Structural information about r(CUG) repeats could be used to inform small molecule design. Various structural studies provide insight into the nature of the UU pairs present in expanded r(CUG) repeats.[111] Crystal structures of oligonucleotide models support computational and thermodynamic investigations in which r(CUG) repeats fold into a hairpin that displays regularly repeating 1 × 1 nucleotide loops composed of a UU mismatch.[112−114] NMR spectroscopy and molecular dynamics investigations of a single r(CUG) motif (5′CUG/3′GUC) revealed that the UU pair samples multiple



K_d (nM)						
Adenine riboswitch	200	n/a	4	2000	n/a	n/a
Guanine riboswitch	469	4	17	n/a	20	100

Figure 13. The structures of adenine and guanine analogs that bind to purine riboswitches.[73−77]

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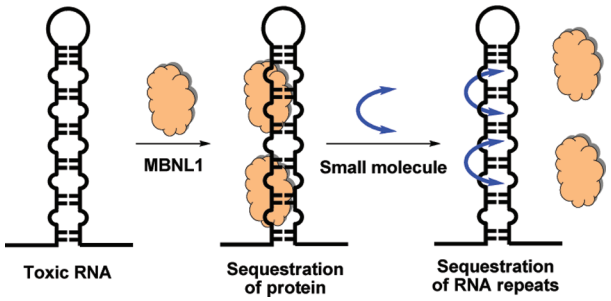

Figure 15. Therapeutic strategy using small molecules to inhibit a toxic RNA–protein complex.

conformations containing zero, one, or two hydrogen bonds.[102] The structure with a single hydrogen bond is the most populated one, but the UU pair interconverts among zero, one, and two hydrogen bond pairs without breaking the loop closing pairs.[115] The observation that the UU pair adopts an ensemble of conformations may have implications in the molecular recognition of ligands by expanded r(CUG) repeats. Below, we summarize efforts by various research groups to design small molecules that target the RNA repeats that cause DM1 and DM2. The strategies that they employ, however, could be applied to other types of RNA repeat expansions.

Targeting r(CUG) Repeats That Cause DM1. A resin-bound dynamic combinatorial library (RBDCL) was screened for binding to r(CUG) repeats.[116] The library, containing a theoretical 11,325 members, was created from 150 resin-attached, cysteine-containing peptides and an identical set of solution-phase peptides. Thus, the resin-bound cysteines and the solution-phase cysteines can form a disulfide bond. The library was incubated with a fluorescently labeled RNA containing r(CUG) repeats. Four disulfide dimers from the combination of the lead monomers (**32**–**35**, Figure 16) were identified with low micromolar binding affinities (K_d = 9.6 − 18 μM). The compounds also inhibit the r(CUG)-MBNL1 interaction with K_i's ranging from ∼5.5 to 10 μM in the presence of competitor tRNA (Figure 16).

Using the crystal structure of a short r(CUG) repeat,[113] Baranger, Zimmerman, and co-workers rationally designed a "stacked intercalator" ligand (**36**) that consists of a well-known acridine DNA intercalator connected to a triaminotriazine unit (Figure 16).[117] The triaminotriazine forms Janus–Wedge type binding with the minor or major grooves of UU or TT mismatches. The optimal distance between the acridine and triaminotriazine was determined by studying a small library of dimers. The optimal ligand contains a four-methylene group linker (**36**). Compound **36** binds to nucleic acids containing d(CTG) and r(CUG) repeats similarly (K_d ≈ 400 nM). It does, however, show selectivity against other mismatches: **36** binds

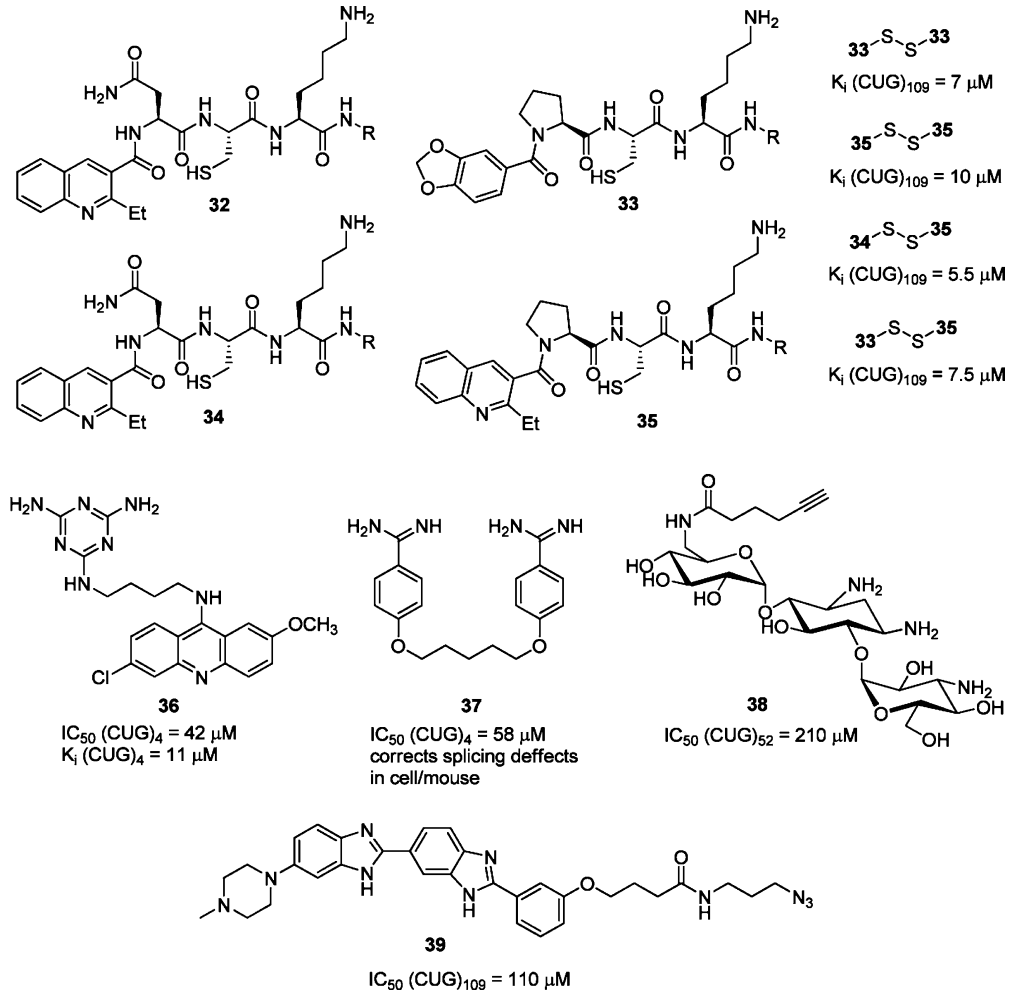

Figure 16. Structures of the small molecules that inhibit the r(CUG)-MBNL1 interaction with IC_{50}'s in the micromolar range.[116–118,127–129]

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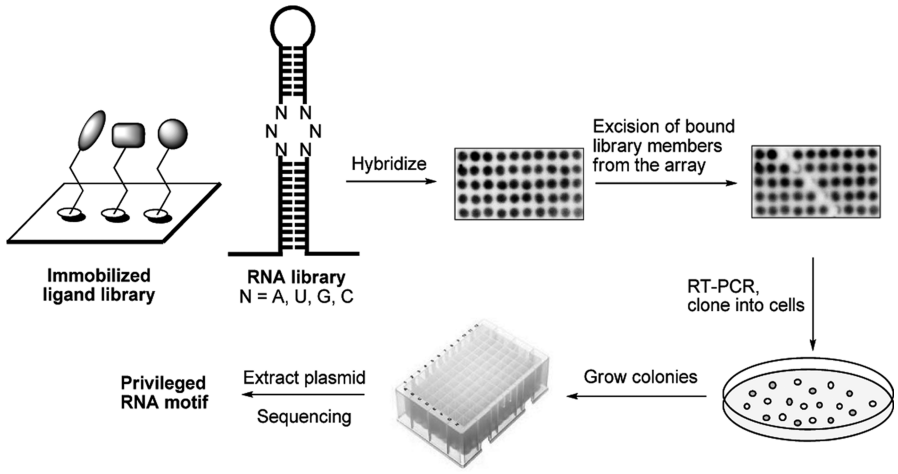
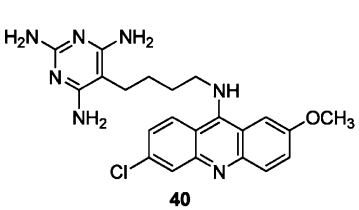


Figure 17. Two-dimensional combinatorial screening (2DCS) selects privileged RNA motifs for a specific ligand by screening a nucleic acid library and a small molecule library simultaneously.[119−126]

13-, 169-, and 85-fold more weakly to single C-C, A-A, and G-G mismatches in DNA, respectively, and 6-, >143-, and >143 times more weakly to single C-C, A-A, and G-G mismatches in RNA, respectively. The ligand also inhibits the r(CUG)-MBNL1 interaction in the presence of competitor tRNA with a K_i in the micromolar range.

Berglund and co-workers screened 26 small molecules with known affinity for nucleic acids *via* a gel shift assay.[118] Among the screened compounds, pentamidine (**37**; Figure 16) disrupts the r(CUG)-MBNL1 complex with an IC_{50} of 58 μM. Additional studies revealed that **37** partially rescues mis-splicing in a DM1 mouse model when mice were dosed with 25 mg/kg twice a day. However, increasing the dosage to 30 mg/kg twice daily was toxic.[118]

A two-dimensional combinatorial screening (2DCS) method was developed by Disney and co-workers in an effort to rationally design small molecules that target RNA.[119−126] The microarray-based method screens chemical and RNA spaces simultaneously by immobilizing a small molecule library on a microarray and hybridizing it with an RNA library that displays a discrete secondary structure element (Figure 17). Utilizing 2DCS, it was determined that an alkynyl derivative of kanamycin A (**38**, Figure 16) prefers to bind pyrimidine-rich internal loops such as those present in the RNAs that cause DM1 an DM2. The **K** module was then modularly assembled onto a peptoid backbone to improve affinity, specificity, and potency (Figure 19).[127,128] A small library of dimers was synthesized in order to determine the optimal distance between **K** ligand modules; that is, which distance affords the same periodicity in the small molecule as in r(CUG) repeats such that two adjacent internal loops are bound simultaneously. Modularly assembled compounds have the general structure **nK-m** where **nK** indicates the valency of the kanamycin derivative and **-m** indicates the number of spacing modules, or distance, between the **K**'s (Figure 19). The optimal compound, **2K-2**, displays two kanamycin derivatives (**2K**) separated by two propylamine spacing modules (**-2**).[127] Interestingly, the increase in potency afforded by increasing the valency of the modularly assembled compounds is much larger than the corresponding increase in affinity (multivalent effect). It is likely that the increase in observed potency is due to surface area affects; that is, the peptoid backbone sterically blocks MBNL1 from binding.



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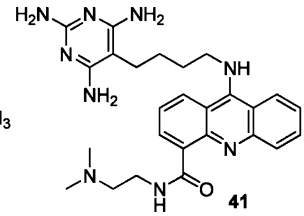

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DM2	IC_{50} (CCUG)$_6$ = 40 μM K_i (CCUG)$_6$ = 3.4 μM	IC_{50} (CCUG)$_6$ = 35 μM K_i (CCUG)$_6$ = 3.0 μM
DM1	IC_{50} (CUG)$_{12}$ = 37 μM K_i (CUG)$_{12}$ = 5.5 μM	IC_{50} (CUG)$_{12}$ = 118 μM K_i (CUG)$_{12}$ = 18 μM

Figure 18. "Stacked intercalator" ligands inhibit formation of the DM1 RNA-MBNL1 and the DM2 RNA-MBNL1 interactions.[130]

A second series of compounds was designed on the basis of a previous study in which it was determined that Hoechst 33258 binds to 1 × 1 nucleotide internal loops, such as those found in r(CUG) repeats.[129] A Hoechst derivative that displays an azide (**39**, Figure 16) was synthesized such that it could be modularly assembled. Analogous to the **nK-m** studies described above, the *in vitro* potencies of a small library of dimers were measured in order to determine the optimal distance between **H** ligand modules (Figure 19). In this case, the optimal distance is four propylamine spacing modules, affording **2H-4**. The corresponding trimer, tetramer, and pentamer (**3H-4**, **4H-4**, and **5H-4**) were then synthesized and tested (Figure 19). They inhibit MBNL1 binding and displace MBNL1 from r(CUG) repeats with nanomolar potencies in the presence of competitor tRNA.[129] The higher valency compounds, **4H-4** and **5H-4**, bind to r(CUG) repeats with greater affinity and specificity than MBNL1.[129] Bonuses in inhibition due to the multivalent effect were observed, as was also observed with **nK-2** ligands.[127,129]

For both the **nK-2** and **nH-4** compounds, a subset of the designed modularly assembled compounds (**4K-2**, **4H-4** and **5H-4**) binds r(CUG) repeats with >10-fold higher affinity and with >10-fold higher specificity than MBNL1. These binding properties are important, as a small molecule has to compete with MBNL1 for binding to r(CUG) repeats; that is, the ligands must be either higher affinity than MBNL1 or should be sufficiently cell permeable such that they are present at much

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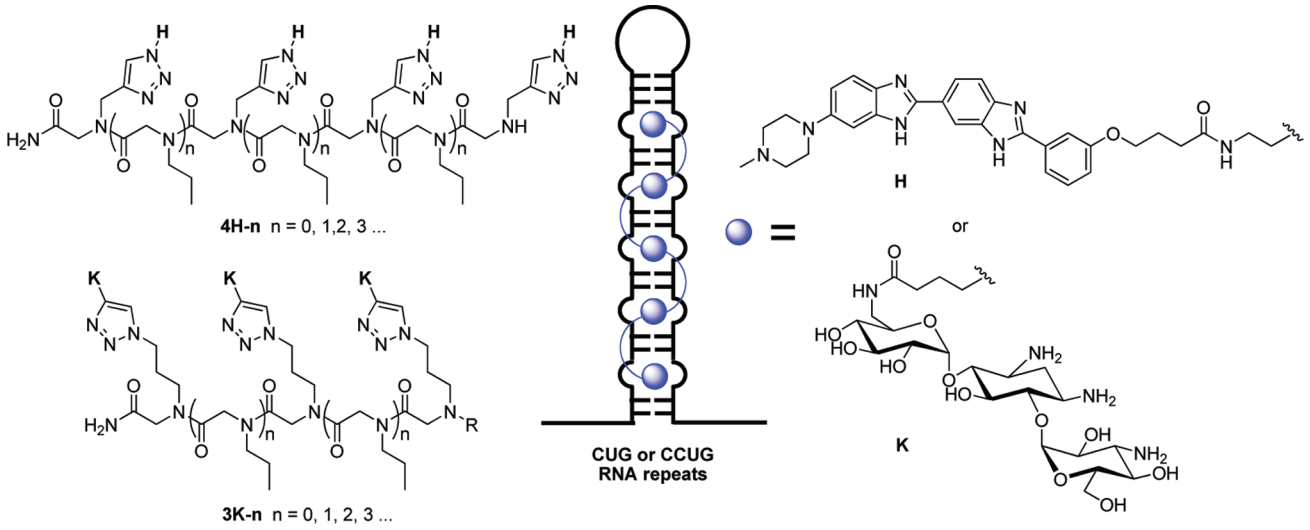

MBNL1-DM1 (CUG) complex						MBNL1-DM2(CCUG) complex	
	K_d (μM)	IC_{50} (μM)		K_d (μM)	IC_{50} (μM)		IC_{50} (μM)
H	0.13	110	**K**	1	>200	**K**	100
2H-4	0.1	1.1	**2K-2**	0.05	0.6	**2K-4**	0.089
3H-4	0.065	0.96	**3K-2**	0.02	0.12	**3K-4**	0.0016
4H-4	0.035	0.39	**4K-2**	0.004	0.02		
5H-4	0.013	0.22					

Figure 19. Modularly assembled ligands that target expanded r(CUG) and r(CCUG) repeats inhibit the formation of the DM1 RNA-MBNL1 and the DM2 RNA-MBNL1 complexes, respectively.[127−129]

higher concentrations than MBNL1 in a biological system. The former is more desirable as requiring a lower effective dose could minimize nonspecific binding events and thus toxicity.

Targeting r(CCUG) Repeats That Cause DM2. As mentioned above, DM2 is caused by an expansion of r(CCUG) repeats, and its disease mechanism is similar to that of DM1. Thus, a similar approach to target r(CUG) repeats by Baranger, Zimmerman, and co-workers was also used to design ligands that bind r(CCUG) repeats. The group optimized their "stacked intercalator" ligands that were used to target r(CUG) repeats (**40**, **41**, Figure 18).[117,130] Compound **41** selectively inhibits the r(CCUG)-MBNL1 interaction over the r(CUG)-MBNL1 interaction (Figure 18). By examining the affinities of the related conjugates for the r(CCUG) repeat, it was determined that the acridine imparts affinity while the nature of the wedge motif imparts selectivity.[130]

Disney and co-workers also used their modular assembly approach to design compounds that target r(CCUG) repeats. Again, a series of dimeric and trimeric peptoids displaying **K** (Figure 19) with different distances between ligand modules were synthesized and investigated.[128] Not surprisingly, the optimal distance between the **K**'s is longer for the r(CCUG) repeats (**nK-4**) than the r(CUG) repeats (**nK-2**) due to the difference in the sizes of the internal loops (2 × 2 nucleotide vs 1 × 1 nucleotide). The compounds are potent inhibitors of the r(CCUG)-MBNL1 interaction: IC_{50} values of 89 nM for **2K-4** and 1.6 nM for the trimer **3K-4**. The compounds bind with high affinity: **2K-4**, and **3K-4** bind with K_d's of 50 and 8 nM, respectively.[128] **2K-4** and **3K-4** are higher affinity for r(CCUG) repeats than MBNL1, by 2-fold and 15-fold, respectively. The trimer, **3K-4**, is also selective. It recognizes RNAs with multiple copies of the 5′CCUG/3′GUCC (DM2) motif with higher affinity than RNAs in which the 2 × 2 nucleotide internal loop is modified to contain multiple copies of 5′CCCG/′3GCCC or 5′CUUG/3′GUUC. As was observed with other modularly assembled compounds,[127,129] the increase in affinity afforded by multivalency is much smaller than the increase observed in potency,[128] suggesting that the amount of surface area sequestered by a ligand is important.

The **K** module was used to bind the pyrimidine-rich loops formed by expanded r(CUG) and r(CCUG) repeats.[127−129] Interestingly, specificity was achieved by optimizing the distance between **K**'s. Even though the **K** module binds the 5′CCUG/3′GUCC motif found in DM2 with higher affinity than to the 5′CUG/3′GUC motif found in DM1 (Figure 19),[127−129] modularly assembled compounds can be engineered to bind more tightly to the DM1 RNA than the DM2 RNA and *vice versa*. Specifically, **3K-2** is selective for the DM1 RNA over the DM2 RNA by 3-fold, while **3K-4** is selective for DM2 RNA over the DM1 RNA by 20-fold. Taken together, specificity can be affected by as much as 60-fold by changing the spacing between RNA-binding modules.[127−129] The ability to fine-tune RNA-binding specificity by altering the distance between ligand modules can provide opportunities to use both optimal and suboptimal RNA motif-ligand interactions[131] to design high affinity, selective modularly assembled ligands that target a variety of cellular RNAs.

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CONCLUSIONS

Targeting RNA with small molecules is a significant challenge. Very few clinically used drugs elicit their effects by modulating RNA function outside of the bacterial ribosome. One of the major issues with this may be the relative expression of RNAs in a cell. For example, the ribosome constitutes the vast majority of cellular RNA while noncoding RNA collectively constitutes < 5% of total cellular RNA. Thus, targeting one noncoding RNA selectively is challenging as described previously.[114]

In the past few years, however, progress has been made to develop methods to design small molecules that bind to RNA with high affinity and specificity. Computational virtual screening methods based on information about RNA-ligand interactions have yielded new RNA binding scaffolds. Several novel synthetic peptides and modularly assembled small molecules have been identified to target RNA with good affinity and specificity. While new high-throughput screening methods are still highly desirable, newly developed screening methods, such as fragment-based screening, NMR methods, and 2DCS, can facilitate the discovery of novel RNA-binding ligands and new RNA targets. Perhaps by merging these approaches with computational studies on RNA genomic secondary structure,[132,133] one can quickly design small molecules to target a variety of unexploited RNA drug targets in genomic sequence.

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