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Structure based approaches for targeting non-coding RNAs with small molecules

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Abstract

The increasing appreciation of the central role of non-coding RNAs (miRNAs and long non coding RNAs) in chronic and degenerative human disease makes them attractive therapeutic targets. This would not be unprecedented: the bacterial ribosomal RNA is a mainstay for antibacterial treatment, while the conservation and functional importance of viral RNA regulatory elements has long suggested they would constitute attractive targets for new antivirals. Oligonucleotide-based chemistry has obvious appeals but also considerable pharmacological limitations that are yet to be addressed satisfactorily. Recent studies identifying small molecules targeting non-coding RNAs may provide an alternative approach to oligonucleotide methods. Here we review recent work investigating new structural and chemical principles for targeting RNA with small molecules.

Introduction

Exploiting RNA as a drug target has been strongly advocated in antiviral and antibacterial research for > 25 years (reviewed in [1,2]). The exploding awareness of non-coding genes in human disease [3] has prompted renewed interest in the discovery of drug-like chemistries that target RNA in a sequence and/or structure-specific manner. Despite the considerable skepticism as to whether RNA can be targeted specifically using drug-like molecules, several lines of evidence suggest the challenges are not unsurmountable [4,5]. Most ribosomal binding drugs have unattractive physical, chemical, pharmacological and synthetic characteristics, but they are mainstay of antibacterial treatment. Studies of riboswitches, prime drug targets for antibacterial and anti-fungal treatment [1,5], show that small metabolites can be recognized by RNA with exquisite specificity. Finally, many studies on aptamers, RNA molecules selected to bind small molecules, demonstrate that RNA can be readily selected to recognize small molecules with greater sensitivity to fine details of the chemistry than antibodies [6*].

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In this review, we explore recent progress in non-oligonucleotide strategies to target non-coding RNAs with a focus on the first attempts at targeting microRNAs. We show how high resolution structures can guide development of specific inhibitors targeting repeat expansions and highlight progress in targeting RNAs of infectious organisms using new screening methods, and ligand libraries which are helping to identify less cytotoxic, more drug-like inhibitors which directly bind their RNA target.

Targeting the microRNA biogenesis pathway

Mature microRNAs (miRNAs) are small non-coding RNAs (21–23nt) which recognize specific mRNA sequences within the 3' untranslated region of many open reading frames to post-transcriptionally repress gene expression [7]. Over or under-expression of specific microRNAs is associated with many chronic diseases, and the concentration and distribution of circulating microRNAs provide diagnostic tools in cancer [8], suggesting that targeting of these RNAs might have therapeutic benefits. Inhibition of the mature form of the microRNA can be pursued using oligonucleotides, but the biogenesis of mature microRNAs provides multiple avenues for inhibition by different chemistries [9] towards the development of new cancer therapeutics [10].

The first attempts at targeting miRNA biogenesis with small molecules relied on cell-based screens and *in vivo* reporter systems [11]. These ligands were found to work upstream of pri-miRNA processing, the first step in the biogenesis cascade, perhaps through effecting transcription [11]. A similar result was found in screens against the liver associated miR-122 [12]. Both studies showed that selective inhibition of mature miRNA production has the potential for therapeutic development, but neither study included direct investigation of binding to putative miRNA target leaving the actual molecular targets of the ligands unidentified. These results are not unexpected from cell-based screens [13–19]; particularly with libraries biased to contain molecular scaffolds known to target cancer cell [14*] or generic ligands for RNA [16*]. Demonstrating mechanistic links and identifying clear targets in complex, highly integrated cellular pathways like miRNA biogenesis require extensive experiments and stringent controls.

For these reasons, successful targeting of miRNAs might profit from a bottom-up approach: the choice of a specific molecular target, the identification of inhibitors from biochemical assays and building up cellular activity during subsequent development steps. In this context, a structurally and biochemically attractive target within miRNAs is the pre-miRNA hairpin loop, a 70–72 nucleotide structure generated by Drosha cleavage, containing at least one bulge or internal loop region capped by a mostly single stranded apical loop [20]. These loops act as dynamic regulators of microRNA maturations, are landing pads for multiple sequence specific regulatory RBPs and are often evolutionarily conserved, indicating strong functional roles [21,22]. A small molecule that bound specifically to this stem-loop could affect miRNA production, a hypothesis supported by proof of principle studies [23,24].

A number of aminoglycosides and other small molecules have been identified to bind to the terminal loop region of pre-miRNAs miR-10b [25*], miR-21 [26] and miR-155 [27*] (Figure 1) but it is very questionable whether these interactions are specific [28]. A new method called Inforna was recently developed to identify potential ligand binding sites within

miRNA precursors [29**]. Inforna is an integrated computational and medicinal chemistry method that generates lead compounds by identifying ligands which bind common structural motifs in the target. The authors computationally screened all human pre-miRNA secondary structures deposited in miRBase against a database of known ligand interactions which bind specific RNA motifs (apical loops, bulges, and internal loops). This method was used to probe >5,000,000 potential interactions from 1,048 miRNA precursors with ~7,000 different motifs and 792 known RNA motif-small molecule interactions contained in an in house database. Ligands predicted to bind to either the Drosha or Dicer cleavage sites were assayed in MCF-7 cells for changes in miRNA expression. The best compound, as determined by a derived fitness score, disrupted *in vivo* processing of mature miRNA-96 and *in vitro* Drosha processing of pri-miRNA-96. However, the primary ligand scaffold within the library resembles derivatives of Hoechst dyes (Figure 1), which bind to many RNAs non-specifically, including other pre-miRNAs hairpins [27]. Thus, direct binding affinity measurements towards the suspected miR-96 target must be conducted and the mechanism of action in cells remains to be established and carefully validated.

A different approach to targeting miRNA biogenesis was initially reported about five years ago and recently updated [30,31*]. The authors identified a set of peptidomimetic ligands which target the pre-miRNA-21 terminal loop region [30]; a well-established and validated cancer target. Peptoid microarrays were screened against a shortened 29 nucleotide pre-miR-21 terminal loop and a control pre-miR-21 loop with scrambled terminal loop (Figure 2A **sequence I and II**). Over 800 initial hits were found; the best binding ligand (Figure 2A **compound 1**) had a dissociation constant (K_D) of 15 μ M and was approximately 4 times selective over the control target (K_D ~71 μ M). These initial results stimulated the authors to search for contributions to affinity and found that changes in methyl pyridine position improved binding by nearly an order of magnitude (Figure 2A **compounds 2 and 3**). An expanded peptoid library was designed in a recent follow up study based on the initial best ligand and inhibition of *in vitro* Drosha processing was observed, specific to pri-miR-21 over pri-miR-16 [31*]. The new most potent peptoid appears to bind more weakly than the previous best ligand (Figure 2A **compound 4**) but has better (although still limited) selectivity toward pre-miR-21.

In both peptoid studies, the authors used footprinting with RNase T1 and in-line cleavage assays to clearly show a change in RNA structure upon binding the peptoid [30,31*]. However, it remains unclear how the peptoid binds relative to the target; or if the peptoid binds in a single or multiple orientations and locations. In a recent report, the authors used NMR to characterize the free terminal loop of the miR-21 precursor [32*] (Figure 2B); the structure appears very flexible. Peptoids were used in the past to target specific RNA hairpin structures [33] but their flexibility limited optimization to generate specific inhibitors. Optimizing a flexible ligand against a flexible target would be very challenging.

The work on miRNAs summarized here is driven by a clear biological rational and pharmaceutical potential, but we are just starting to learn how to target precursor miRNAs. High resolution structural work of different pre-miRNAs and, especially, pre-miRNA-ligand complexes are very much needed to fully understand how to target this class of RNAs.

Rational design of ligands which recognize RNA repeat expansions

Repeat expansions found in both mRNA transcripts and terminal ends of telomeres are implicated in many neurological/neuromuscular disorders and even certain cancers [34]. Much of the investigation in targeting repeat expansions has focused on disrupting the CUG repeats found in the DMPK gene to relieve symptoms of myotonic dystrophy. The first crystal structure of r(CUG)₆ repeats [35] showed a long, mostly A-form helix, modulated by U-U mismatches at specific and uniform distances (Figure 3A). The same group screened a library of known RNA binding compounds against a protein-RNA complex of MBNL and r(CUG) repeats and measured disruption in complex formation. They discovered that pentamidine is a competitive ligand that disrupts formation of a specific protein (MBNL) complex with the repeat with IC₅₀ of 58 μM [36]. However, the initial screening assay only indirectly detected RNA-ligand binding by measuring the disruption of the protein-RNA complex. The authors recently reported being unable to measure direct ligand binding between the pentamidine ligand and the RNA target, suggesting an alternative mechanism of action. [37]

The Disney group has also been very active in identifying inhibitors of different RNA repeats including transcripts involved in both myotonic dystrophies (DM1 and DM2), fragile X-associated tremor ataxia (FXTAS), and, most recently amyotrophic lateral sclerosis (ALS) [38–51]. The group recently reported the first crystal structure of the CCUG tetranucleotide repeat expansion involved in myotonic dystrophy type 2 (Figure 3B) [47]. This prolific program relies on the development of two-dimensional combinatorial screening method [52] coupled with the establishment of structure-activity relationships through sequencing (StARTS) [53] to rapidly identify ligands which bind a variety of structured RNAs by leveraging similarities in binding sites. Similar targets with similar structures unsurprisingly recognize similar ligands. This is highlighted by the same ellipticine derivative binding a common 1×1 GG mismatch found in the r(CGG)_{exp} repeat associated with FXTAS [44] and the r(GGGGCC)_{exp} repeat associated with the c9FTD/ALS gene: with comparable affinities (Figure 3C) [50]. Using similarities in ligand binding can overcome optimization challenges with respect to toxicity, as described in a new paper targeting repeat r(CUG) expansions [54], but specificity might be difficult to achieve starting from scaffolds that are promiscuous by design.

Early work targeting r(CUG)_{exp} repeats originated with triaminotriazine-acridine conjugates specifically binding U-U mismatches [55] which were poorly water soluble and exhibited an unsurprisingly high level of cytotoxicity, even after optimizations [56,57]. Related compounds were discovered in a screen at Ciba-Geigy nearly 20 years ago and immediately abandoned because of toxicity [58]. The authors recently compared the structures of r(CUG) repeats with that of the HIV-1 frame shifting RNA element bound to a bisamidinium ligand [54,59]. Based on the structure, they predicted that coupling two triaminotriazine-based recognition units onto the bisamidinium fragment would improve binding towards r(CUG) repeats while reducing unwanted toxicity (Figure 3A). Testing different linkage lengths, the authors found that adding a single methylene group improved binding to 8 μM and provided some specificity against tRNA, GST-tagged MBNL1, HIV frame shift RNA, and r(CCUG)₈ sequences. The best ligand also inhibited the formation of r(CUG)₁₂-MBNL1 complex with

an IC₅₀ of 115 μ M *in vitro* and a series of in cell and fly models showed the small molecule could rescue the normal phenotype. Importantly, the switch to the bisamidinium fragment, compared to acridine, dramatically reduced toxicity; no cell death was observed and doses of between 50 and 100 mg/kg were tolerated in mouse.

These papers suggest that designing Janus-wedge like compounds to target other RNA helical mismatches could provide a general approach for targeting repeat RNAs. It might be better, though, to start with compounds that are both non-toxic and specific from the beginning. A new paper targeting telomeric repeat containing RNAs (TERRA) using a fragment based screening approach [60**] shows that repeat expansions can be targeted with such approach. TERRA molecules contain an average of 34 r(UUAGGG) repeats which fold into G-quadruplexes and function as scaffold of telomeric heterochromatin in cancer cells. The authors designed a library of fluorinated fragment probes which were screened using ¹⁹F NMR and identified 20 hits from a library of 355 compounds, 7 of which were able to specifically recognize the parallel propeller structure of RNA G-quadruplexes (Figure 3D). Similar to other fragment-based screens, the confirmed hits had relatively weak binding, K_D=1–2mM; yet were selective over phenylalanine tRNA (tRNA^{Phe}) which has comparable size but different structure. To further rule out generic nucleic acid binding, the authors measured affinities of the top hits towards duplex B-form DNA structure (d(GCAATTGC)). Only two of the seven hits bound B-form DNA duplex. Finally, the authors found all the RNA G-quadruplex hits also bound to the DNA G-quadruplex structure. This result suggests that the ligands can select and have a strong preference for the parallel propeller-like conformation found in telomeric sequences. Additionally, the work shows that small halogenated fragment molecules with good ligand efficiencies can be incorporated into libraries when targeting RNA. It is hoped that the authors will follow up with fragment growing/linking and structure based work to examine how these fluorinated compounds interact with RNA.

New antivirals and antibacterials

Natural products binding ribosomal RNAs represent the most advanced example of RNA-binding pharmacology, a subject that has been extensively reviewed [4,61]. The internal ribosome entry site (IRES) of hepatitis C virus (HCV) and the transactivation response element (TAR) of human immunodeficiency virus (HIV) are other popular RNA targets for antiviral development [1,2,62] and high resolution structures were paramount to developing potent and specific ligands HCV IRES and HIV TAR RNAs [63,64].

A number of new approaches have been described to identify RNA binding ligands for these RNAs, including *in silico* screening [65], small molecule microarray approaches [66**], FRET based assays [67*] and NMR fragment based work. It is important to note that any screening library is just as important as the screening method. Thus, a recent paper combined small molecule microarray screening with a library of 20,000 chemically diverse, drug-like compounds to identify new small molecules which bind to the HIV-TAR RNA [66**]. The library was unbiased with respect to known nucleic acid binding scaffolds and control for non-specific binding was achieved by comparing results to the unrelated pre-miR-21 hairpin. These controls resulted in a reduced screening hit rate (0.02%), much more

similar to traditional protein high-throughput screens. Interestingly, nearly all hits had the same general scaffold. The authors followed up with direct quantitative binding studies using a 2-aminopurine titration assay which gave binding affinities in the low micro-molar range. They completed docking studies of their best compound to six TAR structures and concluded that binding was driven primarily by van der Waals forces, hydrogen bonding and hydrophobic interactions rather than electrostatic interactions. Finally, cell based work showed that the best compound could inhibit HIV-induced cytopathicity with EC₅₀ of 28 μ M and low cytotoxicity up to 1 mM. This work highlights that safe and specific small molecules can be identified with a format quickly amenable to many different targets and scalable for larger libraries.

A different approach to high-throughput screening focuses on drug-like fragments to be built up into larger molecules. The smaller sized fragments increase efficient sampling of chemical space, improve ligand efficiency and reduce steric clashes. Fragments which bind proximally are linked together or grown using a variety of combinatorial and SAR methods. This approach has rapidly generated approved therapeutics [68,69] and recently has been applied to a handful of RNA targets including bacterial riboswitches [70,71^{*}], and viral RNAs [72–74^{*}].

The RNA promoter of influenza A is an extremely well conserved structure required for viral replication. By screening a library of >4,000 drug-like compounds, a new small molecule was identified which bound with approximately 50 μ M K_D. The structure of the complex identified strong interactions arising from a dimethoxy group, while weaker contacts between a piperazine ring and RNA (Figure 4). The piperazine ring may not be needed and could simply provide non-specific binding. Interestingly, the winning ligand was non-toxic at concentrations up to 500 μ M and inhibited influenza A and B viral strains H1N1, H3N2 and PNM with approximate EC₅₀ values of 71 μ M, 275 μ M, and 113 μ M respectively. Despite the weak cellular activity, the ligand is efficient (small size per free energy of binding) and optimizations of the ligand could improve inhibitory effects. The structure provides a good starting point for this process.

The combination of fragment screens and structural investigation could lead down the path of many successful programs in pharmaceutical fragment screening, especially because the hits obtained from fragment based methods have better drug-like properties and ligand efficiencies compared to the lead obtained from other methods described in this review. Researchers wishing to target RNA and design compound libraries will need to pay closer attention to the “frequent hitter” problem, as described for example as Pan Assay Interference Compounds (PAINS) [75], i.e. fragments with low solubility, selectivity or potentially reactive in cells, which will easily give rise to false positives in cell based assays. For example, many fragments and molecules reported within these references contain piperazine or morpholino moieties. It is not clear yet if these represent undesirable “frequent hitters” or a true class of privileged scaffold for RNA targeting to maintain and enrich within a library.

Conclusions

Interest in targeting non-coding RNA using oligonucleotide-based and non-oligonucleotide chemistry has re-surfaced in the past few years. Since nature can specifically target RNA with small molecules, we should be able to turn RNA into a therapeutically valuable target as well. However, developing structured RNAs into successful drug targets remains a very significant challenge which has not been met thus far. Targeting RNA is particularly challenging because of the structural, dynamic, and chemical characteristics of the target molecule. Additionally, we still only have a rudimentary understanding of the principles of RNA recognition. To compensate and increase hits, classical “rules” used for protein targeting [76] have often been ignored while targeting RNA in order to identify ligands at any cost. This approach has most certainly led down blind alleys and compromised potential drug development opportunities. A handful of sustained programs demonstrate that it is possible to find small (<400 Da) molecules that bind to RNA tightly and specifically, but required the synthesis of >500 compounds [77,78], as is common with proteins. There is a strong need for a proper set of guiding principles, to focus and identify an RNA binding chemical space. Finding these principles will require searching beyond known RNA binding ligands. We are nonetheless hopeful that the increasing appreciation of the potential importance of RNA as a drug target will lead to increased investigations in this area; especially more structures of small molecule RNA complexes to guide the development of recognition principles.

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Highlights

- Non-coding RNAs provide new therapeutic targets to treat chronic conditions
- Successful targeting by small molecules is dependent on RNA structural features
- New rational methods to identify selective non-oligonucleotide inhibitors

Figure 1.

Many aminoglycosides bind different microRNA precursors (and many other RNAs) with moderate binding affinities; including streptomycin, dihydrostreptomycin, kanamycin A, kanamycin B, neomycin, and guanidinylated kanamycin A and guanidinylated neomycin. Additionally, intercalators and dyes also bind miRNA precursors, presumably near bulge and internal loop regions, and other RNAs.

Figure 2.

A) Peptoid ligands identified to bind the terminal loop of pre-miRNA-21 and four control sequences used in the studies [30,31] (arrows represent reported T1 cleavage sites). Compounds 1–3 were identified first and compound 4 was most selective. The association constants are reported in micromolar units. **B)** Representative structures of the miR-21 precursor terminal loop.

Figure 3.

Different chemistries to target repeat expansion RNAs, generated using a modular approach to target specific mis-matches within a structure. **A)** The crystal structure of r(CUG) repeat and a newly reported less toxic inhibitor generated by scaffolding the triaminotriazine Janus wedge fragment onto the bis-amidinium structure. U:U mismatches are highlighted in green, the triaminotriazine fragments found on linker of (n=0,1,2 methylene groups) with n=1 is the best ligand. **B)** The first reported structure of the r(CCUG) tetranucleotide repeat expansion RNA implicated in myotonic dystrophy type 2 along with Kanamycin A (K) and neamine-like (N) scaffolds which target the CU bulge regions (orange) in a modular fashion. **C)** The common 1×1 G:G mismatches found in the r(CGG) and r(GGGGCC) repeat expansions implicated with Fragile X-Associated Tremor Ataxia and c9FTD/ALS, respectively, with repeat sequences highlighted by brackets. The same ellipticine derivative binds both target molecules with similar affinities. **D)** Fluorinated fragment hits reported to target a G-quadruplex structure found in telomeric repeats. Although weak binders, the fragments can specifically recognize the parallel propeller-like conformation of certain telomeric sequences.

Figure 4.

Structure of influenza A RNA promoter region bound to a small drug-like molecule. The main contacts indicated by strong NOES were between the methoxy groups of the ligand and the adenosine base pairs highlighted in green, a specific interaction distinct from the common, undesirable electrostatic-driven binding of aminoglycosides and many other proposed RNA ligands. The piperazine fragment could be a source of non-specific binding, but also a good place to start to optimize the activity.