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Small Molecule Chemical Probes of MicroRNA Function

Sai Pradeep Velagapudi, **Balayeshwanth R. Vummidi**, and **Matthew D. Disney***

Department of Chemistry, The Scripps Research Institute, 130 Scripps Way, #3A1, Jupiter, FL 33458

Abstract

MicroRNAs (miRNAs) are small, non-coding RNAs that control protein expression. Aberrant miRNA expression has been linked to various human diseases, and thus miRNAs have been explored as diagnostic markers and therapeutic targets. Although it is challenging to target RNA with small molecules in general, there have been successful campaigns that have identified small molecule modulators of miRNA function by targeting various pathways. For example, small molecules that modulate transcription and target nuclease processing sites in miRNA precursors have been identified. Herein, we describe challenges in developing chemical probes that target miRNAs and highlight aspects of miRNA cellular biology elucidated by using small molecule chemical probes. We expect that this area will expand dramatically in the near future as strides are made to understand small molecule recognition of RNA from a fundamental perspective.

Introduction

RNA is essential for many cellular process, from translation [1] to gene regulation [2] to the production of metabolites [3] and viral replication and propagation [4,5]. Aberrant RNA function or expression is also causative of disease. Yet, small molecules that target RNA have been limited mainly to antibacterials that target the ribosome. These compounds serve as invaluable therapeutics and chemical probes that have elucidated the intricacies of translation [6]. There is clear potential for many RNAs, both human and viral, to be targeted with small molecules yet such investigations have been only sparsely reported. One key to advance this area is a fundamental understanding of selective small molecule-RNA recognition events.

Approaches have been developed to identify and design lead small molecules for both protein and DNA [7–9]. Rules have been established to target the DNA minor groove. The eponymous “Dervan Rules” have allowed for the facile design of small molecules that read out the hydrogen bond donor and acceptor patterns displayed by base pairs [10]. Transition state mimicry approaches can facilitate design of enzyme inhibitors; small molecule screening can be used to identify leads for other types of proteins. Substrate mimicry has

*Author to whom correspondence is addressed; Disney@scripps.edu.

been applied to RNA; riboswitches can be targeted with small molecules that mimic the metabolite that they bind.

RNA, however, is generally considered to be “undruggable” with small molecules [11,12]. This perception is due to a variety of factors, including: (i) there are limited data on the molecular recognition of RNA by small molecules that elicit a biological response; (ii) little is known about chemotypes that impart selective recognition of and affinity for RNA; and (iii) perhaps a false perception that all RNA secondary structures are redundant within the transcriptome, making RNA-selective targeting difficult if not impossible.

In this review, we describe the development of RNA-directed chemical probes and lead therapeutics that target microRNAs (miRNAs). Since their initial discovery by Ambrose and Ruvkin in *Caenorhabditis elegans* [13], miRNAs have been discovered in many kingdoms of life. Because of their essential roles, the development of chemical probes that selectively target a miRNA could be extraordinarily powerful. Coupled with RNA-seq, complex cellular networks could be mapped out by boosting or inhibiting miRNA activity.

MiRNA discovery and biogenesis

Subsequent to their discovery, thousands of miRNAs have been identified and annotated in an online database, miRBase [14]. As of July 2014, miRBase contains 30,424 miRNAs from 206 species [14]. Signature miRNA expression profiles have been associated with nearly every cellular process from development to human disease. Thus, there is a large interest in understanding their exact roles in cellular biology, to develop miRNA expression patterns as diagnostics, and to drug miRNAs as therapeutic targets.

After their initial discovery, intense studies were undertaken to understand miRNA synthesis and biogenesis. MiRNAs are transcribed as precursors mainly by RNA polymerase II [15], however some viral miRNAs are transcribed by RNA polymerase III [16]. These precursor miRNAs fold into stem-loop structures encoding the mature miRNA that are capped with 7-methylguanosine at the 5′ end and polyadenylated at the 3′ end [17]. Often multiple miRNA precursors are transcribed on a single transcript, or a primary miRNA (pri-miRNA), that can be several kilobases in length [18]. Precursor miRNAs (including pri-miRNAs) are cleaved in the nucleus by the microprocessor complex between the nuclease Drosha and DiGeorge Syndrome Critical Region 8 protein (DGCR8) [19], affording pre-miRNA(s).

When a pre-miRNA is liberated, it is translocated to the cytoplasm by Exportin-5 (EXP5) and cleaved by the nuclease Dicer, which forms a complex with the Trans Activating Response RNA-binding protein (TRBP), into an miRNA duplex of 21–25 nucleotides in each strand [20]. The duplex, which contains both the active miRNA and a passenger strand, is loaded onto argonaute (AGO) protein to form the RNA-induced silencing complex (RISC) [20]. In most cases, the passenger strand is removed; however, in some instances both strands are involved in silencing [21,22]. The RISC complex binds to 3′ untranslated regions (UTRs) of mRNAs and either induces cleavage of the mRNA (full miRNA-mRNA complementarity) or translationally represses it (partial miRNA-mRNA duplex complementarity) [23].

Once a mature miRNA is produced, a variety of factors control the downstream mRNA it targets (single or multiple messages). The concentration of the miRNA plays a significant role in mediating gene regulation as well as the stability of the miRNA-mRNA duplex, with higher affinity duplexes active at lower concentrations. For instance, if the concentration of a miRNA is lower than the affinity of miRNA-mRNA complex, then its effect on the mRNA is generally considered negligible [24], as validated by Mullakandov and coworkers [25].

Aberrant miRNAs expression is associated with disease

In general, miRNA expression is tightly controlled. Both up- and down-regulation of miRNA expression can be directly attributed to disease [26,27]. Seed-targeting oligonucleotides and antagomirs that knock out miRNA activity and reverse phenotype convincingly show that miRNAs can be causative of disease [28,29].

The mechanisms by which miRNA expression is altered in disease are varied (Figure 1). Often these effects are at the transcriptional level in which there is an altered binding of transcription factors to gene promoters. Tumor protein p53 (p53), myelocytomatosis viral oncogene homolog (MYC) and Twist-related protein 1 (TWIST) have been shown to affect miRNA expression [30–33]. Regulation of transcription factors leads to double negative feedback loop mechanisms in turn controlling miRNA expression [34]. Further, miRNA expression can be regulated at the post-transcriptional level and aberrant regulation is associated with cancer [27]. In principle, each mechanism could be targeted for therapeutic applications (Figure 1). Below, we highlight specific interrogation of these pathways with small molecules to serve as lead therapeutics and chemical probes of miRNA function.

Targeting miRNA transcription factors

It is possible to modulate the expression of miRNAs using small molecules that either activate or repress their transcription (Figure 1). Deiters and co-workers inhibited transcription of miR-21 [35]. MiR-21 is frequently up-regulated in many cancers [36], resulting in down-regulation of pro-apoptotic proteins. Transcriptional inhibitors were identified by completing a small molecule screen in which a 3′ UTR complementary to miR-21 was inserted into a luciferase mRNA reporter [35]. This study identified a diazobenzene **1**, which was optimized to afford **2**, as miR-21 transcriptional inhibitors (Figure 2A). 2 inhibits expression of miR-21 in several cancer cell lines, not transcription globally, suggesting selective modulation.

Small molecules were also discovered to modulate transcription of miR-122, a highly expressed, liver-specific miRNA whose aberrant expression is associated with hepatocellular carcinoma (HCC; down-regulated) [37] and Hepatitis C viral infections (HCV; up-regulated) [38]. Two small molecules that inhibit transcription (**3** and **4**, Figure 2B) and one that promotes transcription of pri-miR-122 (**5**, Figure 2B) were identified using a luciferase reporter system [39]. Compounds **3** and **4** reduced HCV viral load by ~50% at 10 μM. In contrast, **5**, which is a transcriptional inducer of miR-122, boosts miR-122 levels in an HCC cell line and induces apoptosis at 3 μM. Thus, small molecules that target miRNA transcriptional factors could be developed into anti-cancer and anti-viral agents [39].

Targeting RNA biogenesis by inhibiting Dicer and Drosha processing

Many sites within RNAs can be bound by small molecules; however, it is likely that binding to only a percentage of these sites will have a functional consequence. Thus, exquisite selectivity would likely not be a pre-requisite for an RNA-targeting small molecule agent, provided binding to off-target sites are lower affinity and/or the sites are not functional (no biological consequence). In miRNA precursors, sites that are cleaved by the nucleases Drosha or Dicer are functional sites, the inhibition of which would decrease production of the mature miRNA and de-repress downstream mRNA targets (Figure 1).

Maiti and co-workers showed that inhibition of miR-21 processing by Dicer is an effective strategy to affect miRNA biology [40]. Aminoglycoside antibiotics were screened to identify compounds that inhibit Dicer processing, which showed that streptomycin (**6**; Figure 3A) inhibits miR-21 maturation. *In vitro* experiments showed that streptomycin binds directly to pre-miR-21. Further, a luciferase reporter using the programmed cell death protein 4 (PDCD4) 3′ UTR, a known target of miR-21, showed induction of luciferase activity upon addition of streptomycin. These results were further supported by an Annexin V/propidium iodide assay, which showed induction of apoptosis in Jurkat (T cell leukemia) cells.

Duca and co-workers identified small molecule inhibitors of the Dicer processing of pre-miR-372 and pre-miR-373, which are implicated in gastric cancer [41]. A variety of neomycin-nucleobase conjugates were screened to identify binders to the pre-miRNAs, affording **7** (Figure 3A). Compound **7** (25 μM) inhibits maturation of pre-miR-372 and pre-miR-373 and decreases production of large tumor suppressor homologue 2 (LATS2) protein, which induces cytostatic effects. Down-regulation of miR-17-5p was also observed when cells were treated with **7**, which is likely due to structural similarities between the pre-miRNAs.

Recently, we described the development of rational design approach, named Inforna, to target miRNAs in a transcriptome-wide and target agnostic manner [42]. The basic premise of these studies was to leverage data about the RNA motifs that bind small molecules and cross-reference these data to the motifs found in Dicer and Drosha processing sites as reported in miRBase [14]. RNA motif-small molecule interactions were identified and annotated by using two methods developed in our laboratory, Two-Dimensional Combinatorial Screening (2DCS) [43] and Structure-Activity Relationships Through Sequencing (StARTS) [44]. In 2DCS, a small library of RNA motifs, which contains thousands of members, is probed for binding to a library of small molecules under highly stringent conditions. Thus, the optimal RNA motifs that bind small molecules are selected. StARTS is a statistical approach that predicts the fitness of RNA-small molecule interactions (based on affinity and selectivity). Taken together, Inforna integrates the results of 2DCS selections and their subsequent analysis by StARTS, thereby identifying the ideal RNA target for a given small molecule and making the approach target agnostic.

Inforna predicted lead small molecules for 26 miRNAs; ~45% of these interactions inhibited production of the desired mature miRNA in cell lines without lead optimization [42]. Most notable was the small molecule **8** that binds the Drosha site in the miR-96 hairpin precursor.

MiR-96 is over-expressed in various cancers, in particular breast cancer [45,46]. Indeed, **8** inhibits maturation of miR-96 as determined by qRT-PCR of mature (90% at 40 μM), pre-, and pri-miRNA expression levels in MCF7 cells. Further, inhibition of miR-96 de-represses its downstream target, forkhead box protein O1 (FOXO1), and promotes apoptosis. siRNA knockdown of *FOXO1* confirmed that **8**'s mode of action was via the miR-96-FOXO1 circuit. These studies revealed other interesting aspects of miRNA biology. For example, *FOXO1* is a target of miR-96, miR-182, and miR-27a [46]. However, our studies showed that inhibition of miR-96 biogenesis is sufficient to promote apoptosis [42].

Informa predicted other sites in miRNA precursors that could bind **8** including the Dicer sites in miR-433, -301a, -320c, and 320d-1 and the Drosha site in 449c. Binding of **8** to these sites, however, is predicted to be less fit (Figure 4A) than the Drosha site in miR-96. Indeed, transcriptome-wide qRT-PCR profiling showed that **8** selectively inhibits miR-96 maturation (Figure 4B). Interestingly, miR-96, miR-182, and miR-183 are transcribed in a single transcript. Since miR-182 and miR-183 expression levels are unaffected by **8**, there is no cooperativity between nuclease processing of pri-miRNAs. Furthermore, **8** is more selective than a seed-targeting LNA oligonucleotide and an antagomir that target miR-96. Although the perception is that oligonucleotides are exquisitely selective, oligonucleotides can bind to off-targets that have mismatches with high affinity.

Informa also identified a small molecule, guanidinylated Neomycin B (**9**; Figure 3B) that inhibits maturation of pri-miR-10b by binding the Drosha processing site. **9** boosts production of pri-miR-10b *in cellulo* and removes repression of a downstream target, HomeoboxD10 (HOXD10), which functions as a tumor suppressor [47].

Outlook & Future Directions

Since the discovery of miRNAs, a great amount of effort has been expended to define a detailed mechanism for miRNA biogenesis. Likewise, much effort will be required to untangle the intricate cellular biology of miRNAs. The examples above, however, show that miRNA expression can be altered with small molecules, providing promise to expand miRNAs from diagnostic signatures of disease to *bona fide* therapeutic targets. Although RNA is difficult to target selectively and afford a biological response, progress is being made on various fronts. A better understanding of RNA structure and study of RNA-small molecule recognition events could aid in the design of lead small molecules. Given the importance of miRNAs and the promise they hold, high affinity miRNA-binding scaffolds could decode miRNA function and its roles in various cellular pathways. Further, small molecule leads could be developed into therapeutic agents for disease-associated miRNAs and perhaps personalized medicines.

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Highlights

- Challenges associated with selective recognition of RNA by small molecules
- Review of methods to target miRNAs with small molecules
- Downstream biological responses of small molecules that modulate miRNA function
- Future directions in the area to develop lead RNA-directed therapeutics and chemical probes of function

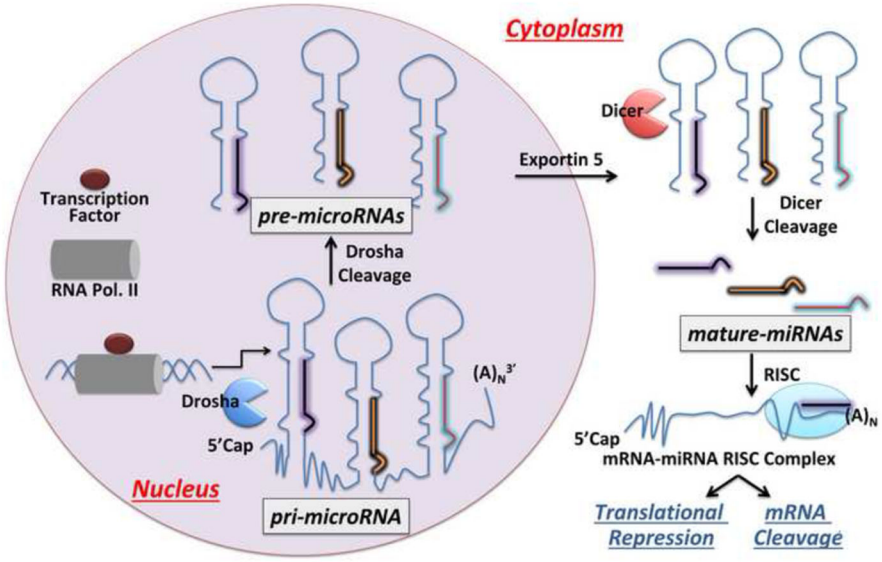

Figure 1.

Biogenesis of microRNAs. MiRNAs are transcribed by RNA Polymerase II into primary miRNA (pri-miRNA) transcripts that are 5′ capped and 3′ poly(A) tailed. The transcribed pri-miRNA is then processed into precursor miRNA (pre-miRNA) by Drosha and exported to the cytoplasm by Exportin-5. Dicer processes the exported pre-miRNA into the mature miRNA. The mature miRNA forms a complex with AGO2 to form the miRNA-mRNA RISC complex, which can either transitionally repress the mRNA or initiate its cleavage.

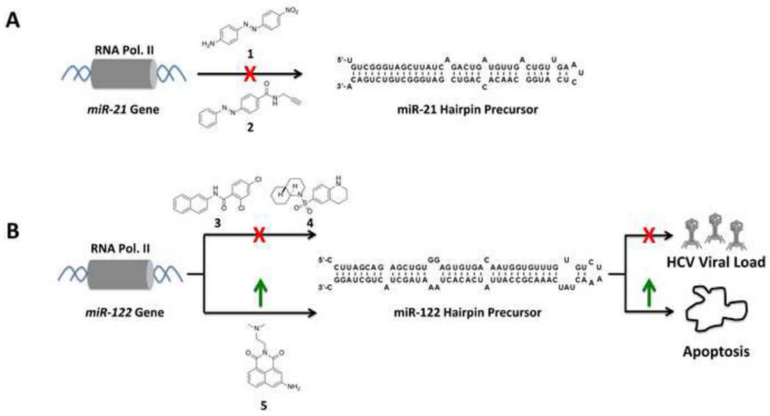

Figure 2.

Targeting transcription of miRNAs. (**A**) Chemical structures of small molecules **1** and **2** that inhibit transcription of miR-21 and the secondary structure of precursor miR-21. (**B**) Chemical structures of small molecules **3**, **4** (inhibitors) and **5** (activator) that affect the transcription of miR-122. The downstream effects of modulating miRNA-122 are also shown.

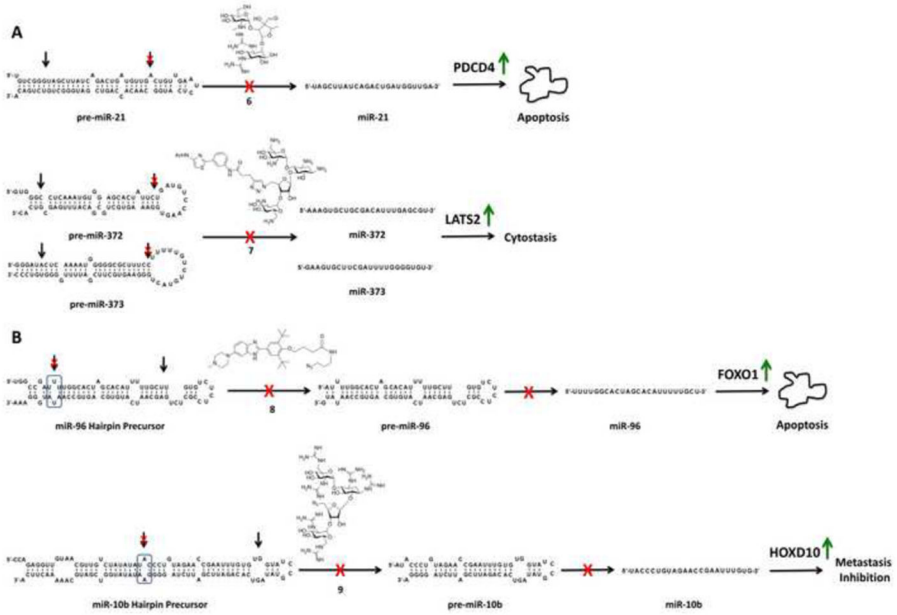

Figure 3.

Chemical structures of small molecules that inhibit Dicer and Drosha processing of precursor miRNAs and the corresponding miRNA secondary structures. (**A**) **6** and **7** inhibit Dicer processing. Inhibition of miR-21 processing by streptomycin up-regulates PDCD4, which induces apoptosis. Inhibition of miR-372 and -373 processing by **7** causes up-regulation of LATS2 inducing cytostatic effects. (**B**) **8** and **9** inhibit Drosha processing of miR-96 and miR-10b, respectively. Inhibition of miR-96 processing up-regulates FOXO1 thereby inducing apoptosis. Inhibition of miR-10b processing induces up-regulation of HOXD10, which can inhibit cell migration and metastasis. Drosha and Dicer processing sites are indicated with arrows.

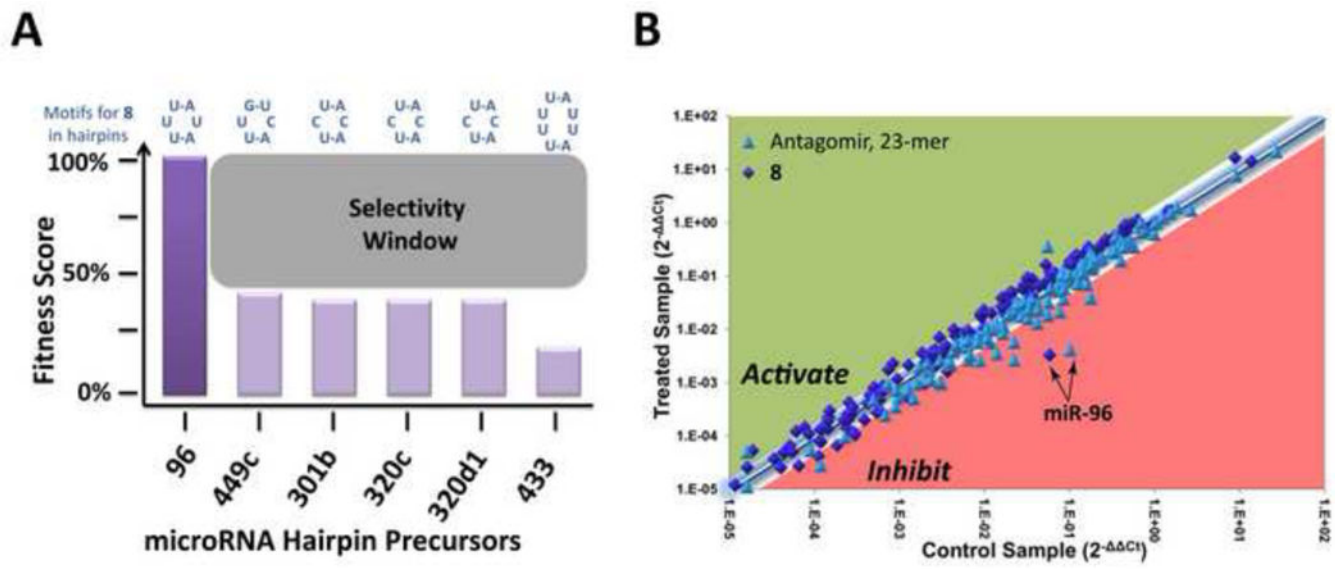

Figure 4.

Compound **8** selectively inhibits miR-96. (**A**) Fitness plot for **8**, which indicates optimal and sub-optimal RNA targets. Selectivity window based on fitness score predicts if a miRNA hairpin precursor can be targeted by **8**. (**B**) Profiling the change in expression of 149 miRNAs upon treatment with **8** (diamond) and an antagomir targeting miR-96 (triangle). Only miR-96 is affected (≥2.5 fold) upon treatment with **8**. However, treatment with an antagomir affects expression of 12 miRNAs (≥2.5-fold).