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# Targeting microRNAs with small molecules: Between Dream and Reality

**Shuxing Zhang**[1,*], **Lu Chen**[1], **Eun Jung Jung**[1], and **George A. Calin**[1,2,*]

[1]Department of Experimental Therapeutics, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA

[2]The Center for RNA Interference and Non-Coding RNAs, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA

### Keywords

microRNA; metastases; targeted cancer therapy; computational modeling; drug discovery; small molecule inhibitors

## INTRODUCTION

Currently, one out of four deaths in the United States is due to cancer, therefore, every major therapeutic advance has the potential of saving many lives. One of the most fascinating molecular oncology discoveries in the last decade is that cancer represents a disease in which alterations in both protein-coding genes and non-coding RNAs named microRNAs (miRNAs) complement each other. Here we focus on a novel idea of using small molecules to target overexpressed miRNAs as a new therapy of human diseases, in particular cancer.

## WHAT ARE MICRORNAS?

MicroRNAs (miRNAs) are non-coding RNAs (ncRNAs) which regulate gene expression [1]. Structurally, they are 19 to 24 nucleotide (nt) long RNAs, processed from much longer primary transcripts (100 to 1000 of nts) arising from hairpin loop structures after successive enzymatic maturation steps (by Drosha in the nucleus and Dicer in the cytoplasm). MiRNAs are involved in a variety of biological processes, spanning from development, differentiation, apoptosis and proliferation to senescence and metabolism. Functionally, miRNAs regulate gene expression in a sequence specific fashion, following incorporation into the multi-protein complex RISC (RNA induced silencing complex), primarily upsetting messenger RNAs (mRNAs) translation and/or stability [2]. Overall, the effect of miRNAs is to silence the expression of the target mRNAs either by mRNA cleavage or by translational repression. However, it has been discovered that miRNAs can also increase the expression of a target mRNA [3]. Each miRNA may target several different transcripts. For instance, it has been demonstrated that a cluster of two miRNAs (namely, *miR-15a* and *miR-16*) can affect the expression of about 14% of the human genome in a leukemic cell line [4]. Conversely, the same mRNA can be targeted by several miRNAs.

[*]Corresponding authors: George A. Calin, Tel: 713-792-5461, Fax: 713-745-4528, gcalin@mdanderson.org and Shuxing Zhang, Tel: 713-745-2958, Fax: 713-794-5577, shuzhang@mdanderson.org.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

## WHY MICRORNAS AS TARGETS FOR CANCER THERAPY?

MicroRNA alterations are involved in various human diseases, including cancer, immune disorders or cardiovascular disorders [5]. Their abnormalities are linked to initiation, progression and metastases of human cancers (Table 1) [6], [7]. The main molecular alterations are represented by variations in gene expression, usually mild but with consequences for a vast number of target protein coding genes. The causes of the widespread differential expression of miRNA genes in malignancy compared with normal cells can be explained by the location of these genes in cancer-associated genomic regions, by epigenetic mechanisms, and by alterations in the microRNA processing machinery. MicroRNA expression profiling of human tumors has identified signatures associated with diagnosis, staging, progression, prognosis and response to treatment [6], [7]. In addition, profiling has been exploited to identify microRNAs genes that may represent downstream targets of activated oncogenic pathways or that are targeting protein coding genes involved in cancer. Specifically in human cancers it was found in at least two independent reports published between 2004 and 2009 that 192 miRNAs were abnormally expressed in cancer cells, including 168 overexpressed miRNAs, meaning that high expression of miRNAs is a hallmark of malignant phenotype. Furthermore, two mouse models strongly suggest that alterations in microRNA expression alone cause a cell to become neoplastic: the miR-155 transgenic overexpressing the oncogenic miR-155 develop acute lymphoblastic/high-grade lymphoma [8], while the knockout model of the tumor-suppressor cluster miR-15/16 develop, as in humans, chronic lymphocytic leukemia [9].

The application of RNA inhibition (defined as the blocking of messenger RNA production or function) in the therapy of human disorders presents two ways: a) the use of miRNAs as therapeutic drugs against messenger RNAs of genes proved to be involved in the pathogenesis, and b) the direct targeting of non-coding RNAs that participate in cancer pathogenesis. The main RNA inhibition agents used till now in pre-clinical and clinical studies include: antisense oligonucleotides (ASOs), ribozymes and the DNAzymes, small interfering RNAs (siRNAs) and short hairpinRNAs (shRNAs), and anti-miRNA agents such as ASOs-anti-miRNAs, and locked nucleic acids (LNA)-anti-miRNAs or antagomirs (Table 2) [10]. However, it is known that there are challenges for the delivery of these non-small molecule agents and their pharmacodynamics and pharmacokinetics properties are not ideal.

## A NEW WAY TO TARGET MICRORNAS – THE USE OF SMALL MOLECULES

Due to the above intriguing facts that miRNAs play crucial roles in cancer and other diseases as well as the challenges for nucleotide analogues, it would be promising to develop small-molecule drugs targeting specific miRNAs (that we named SMIRs) and modulating their activities. We anticipate that this will open a new avenue for targeted cancer therapy.

### Challenges and promises of the approach

RNA molecules have long been neglected as promising drug targets, compared with proteins, because of their structural flexibility and highly electronegative surface. Especially, targeting miRNA with small molecules (Figure 1) is new and perceived as challenging due to the paucity of X-ray or NMR structures of miRNAs for *in silico* drug design, let alone the availability of miRNA-Dicer or RISC complex structures [11]. However, success examples where ligands were designed to target RNA molecules, such as ribosome rRNAs, mRNAs, and viral transactivation response (TAR) RNAs, may provide guidelines to discover miRNA-specific drugs for therapeutic purposes [12].

MiRNAs seem druggable from their secondary structure perspective. The formation of stem loops found in pre-miRNAs and the bulges in miRNA is advantageous for targeting by small

molecules [13]. These structural features not only enlarges the major groove for drug entry, but also partially discloses the internal bases, scattering the local electronegative distribution and providing specificity basis for structure-based drug design, and it has been found that highly positive compounds targeting RNA can easily reach nanomolar (nM) binding affinities [13]. Furthermore, it was shown that microRNAs can target not only messenger RNAs but also DNA and, more recently, proteins. *MiR-373* was found to target promoter sequences and induce gene expression [14], while Eiring and colleagues reported a novel function for miRNAs called "decoy activity" - *miR-328* interacts with heterogeneous ribonucleoproteins hnRNP-E2 regulate RNA binding protein function [15].

Despite those challenges mentioned above, some studies published recently are very promising and have shed insights on the miRNA-targeted drug discovery. Gumireddy et al, after screening over 1000 compounds followed with structure-activity relationship (SAR) analysis, identified the diazobenzene and its derivatives as effective inhibitors against pri-miR-21 formation [16]. It was also reported that small molecule enoxacin (Penetrex) could enhance siRNA-mediated mRNA degradation and promoted the biogenesis of endogenous miRNAs [17]. Unfortunately, either inhibitory or enhancing mechanisms involved, such as drug binding site and specificity, still remain poorly understood. However, these studies undoubtedly provide proof-of-concept for modulation of miRNA activity by small molecules. Instead of using the above pathway-based approaches, where the exact mechanisms are not clear, we target specific miRNAs of interest by employing our integrated drug discovery platform through the synergistic collaboration among computational modellers, microRNA bioogist and medicinal chemists. This innovative and synergistic approach can help us to build the 3D structures of miRNAs and use structure-based design methods to perform lead identification and optimization.

## In silico discovery of RNAs small molecule inhibitors

Drug discovery and development is an expensive and time-consuming process. However, computer-aided approaches [18] has become a promising tool as they can improve the pipelines dramatically in a cost-effective way than traditional strategies for RNA-targeted lead identification [19], [20], [21]. Two docking programs, AutoDock and Dock, have been thoroughly assessed for their capacity of reliably predicting the binding sites and affinities of known ligands in RNA system in which receptor flexibility is considered [19], [21], [22]. Various RNAs can be targeted with small molecules including ribosome, tRNA, mRNA, etc. For instance, upon structure-based virtual screening followed with multiple in vitro binding assays, inhibitors that target HIV-1TAR RNA and bacterial ribosomal A-site have been identified [23]. These agents, including but not limited to marketed drug erythromycin, aminoglycosides derivatives, and neamine mimics, are being investigated in clinical studies,. Thus, similar approaches may also be applicable to the discovery of small molecules targeting miRNA for cancer therapeutics development.

## Small molecule inhibitors discovery targeting miRNAs

In order to identify lead compounds that target miRNAs using structure-based approaches, the accurate determination or prediction of miRNA 3D structures is the top priority. Although RNA crystallography is a challenge. *in silico* 3D structure prediction has experienced significant advances during recent years due to the availability of new experimental data along with enhanced computer power and improved modeling methodologies [24]. MC-fold/MC-Sym [25], for example, has successfully made accurate predictions for double helix region of several pre-miRNAs (*let-7c*, *miR-19* and *miR-29a*), whereas an energy-based *de novo* approach was able to recapitulate noncanonical base pairs observed in native RNA structures [26]. Once the miRNA structure is obtained, molecular docking-based virtual high-throughput screening (vHTS) techniques will be used to enhance

the miRNA drug discovery process based on RNA-compatible scoring functions, in which it is necessary to re-evaluate the electrostatic interaction and solvation terms in accordance with experimental statistics [27]. Figure 1 demonstrated such an idea of our effort in the discovery of novel miRNA small molecule inhibitors.

### Pitfalls for discovery of small molecules as miRNA Inhibitors

However, based on the current knowledge, researchers are still far from being able to design novel and potent molecules modulating miRNA pathways with clear understanding of their mechanisms. Therefore, more structural and thermodynamic information on miRNA-small molecule interactions is clearly needed, for instance, to elucidate their 3D structures and illustrate more detailed mechanisms in which miRNAs regulate the gene expression. The fact that even nano-molar binders but with poor specificity implies the necessity to introduce novel concepts and strategies when targeting miRNAs with small molecules. The cost-effective computational approaches employed in our work can certainly help to achieve this task and accelerate the discovery process.

## THE POTENTIAL IMPACT IS TWICE

As miRNAs overexpression was identified in various health conditions, including heart and autoimmune diseases, the development of new small molecules as targeted therapeutics and as probes for miRNA functional analysis can highly advance public health.

### Development of a new type of cancer therapies targeting miRNAs

Targeting miRNAs for anticancer therapeutics development is very innovative and promising, and it is expected to further our research in the miRNA area and move the field from a hypothesis-driven science toward a clinical application through the synergy of the innovative miRNA studies, medicinal synthesis and the development of state-of-the-art computational drug discovery approaches. The impact of this work is multi-fold. First, the identification of potent small molecule inhibitors targeting miRNA can lead to drug development for targeted cancer therapy. Second, third, the computational effort will help to develop an integrated drug discovery platform, which will be made publicly available (database, methodology and predictors) for knowledge dissemination. Third, the accumulated knowledge and the built platform can be readily applied to other miRNA-targeted or in general RNA-targeted small molecule discovery. We estimate that this development will be dramatically helpful to the scientific community of drug discovery, miRNA biology, computational modeling, biological signaling pathway studies, and many other related areas. A logic approach for better cure of cancer patients is to exploit the huge advances in understanding the genetic nature of cancer and the molecular pathways involved in malignant transformation.

Using these advances and the impressive spectrum of new molecular drugs it is logical to start to design various regimens based on combinations of old and new agents. One way to do this, **the multiplex RNA inhibition targeting strategy,** is by targeting various molecular defects in the multistep pathways of specific cancers by using different RNA inhibition approaches. For example, in aggressive forms of chronic lymphocytic leukemia (CLL) both *miR-21* and *miR-155* are overexpressed [28] and therefore in such patients a combination of small molecules specifically targeting these transcripts in addition to the actual chemotherapy regimens could be envisioned. The second way, **the “sandwich RNA inhibition” strategy,** is to focus with multiple different agents on a major molecular alteration clearly linked to the pathogenesis of a disease. This strategy aims at keeping a specific target under multiple destruction pressures by various mechanisms. This is the case of *miR-372/373* cluster overexpression in testicular germ cell tumors [29] – targeting these

genes with small molecules as well as with antagomirs could represent a new way to treat these patients.

### Development of new tools to explore the function of miRNAs

Understanding the roles of miRNAs in cancer cells is of prime importance at the present time, when a large body of evidence provided strong arguments for the involvement for several hundreds miRNAs in human cancers. The available tool to explore the function of overexpressed miRNAs includes anti miRNAs oligonucleotides (AMOs) that are antisense oligonucleotides targeting miRNAs, looked nucleic acids (LNA) anti-miRNAs that comprise a new class of bicyclic high-affinity RNAs targeting miRNAs and a novel class of chemically engineered oligonucleotides named “antagomirs”. Although with good results the use of all these agents has a major drawback for *in vivo* studies – the huge cost. For example, the use of antagomirs in mouse models of cancers has the disadvantage of a cost of several thousand dollars per two groups of five mice (treated versus non-treated) experiment using a weekly administration for 3 to 5 weeks. Therefore, using the much cheaper small molecules already available but not known to target miRNAs, or new small molecules derived from known ones, will represent an alternative for the scientific community. The identified active hits (or the potential drugs) can be used as probes to study the response of miRNA to the compounds and their biological consequences. Furthermore, the tissue distribution and other pharmacokinetics characteristics for specific small molecules are already known from previous studies (or relatively easy to be performed for new molecules) and this will clearly help in performing *in vitro* and *in vivo* experiments.

## CONCLUSION

Cancer is an enormous threat to our health. Although the existing drugs showed promise in the early treatment, most metastatic cancers are still considered as incurable. Hence, there is an urgent unmet need to develop new potential “breakthrough drugs” for cancer treatment. MiRNAs have been found to play important roles in various cancer development and metastasis. We propose a synergistic and innovative approach to develop a novel microRNA-based technology with wide applications for functional studies and targeted therapy, on the hypothesis that small molecule inhibitors could target miRNAs and that the targeted inhibition will have biological consequences culminating with death of cancer cells. This is linking two exciting areas of miRNAs involvement in human cancers and computational discovery of small molecules that inhibit significant targets important in tumorigenesis.

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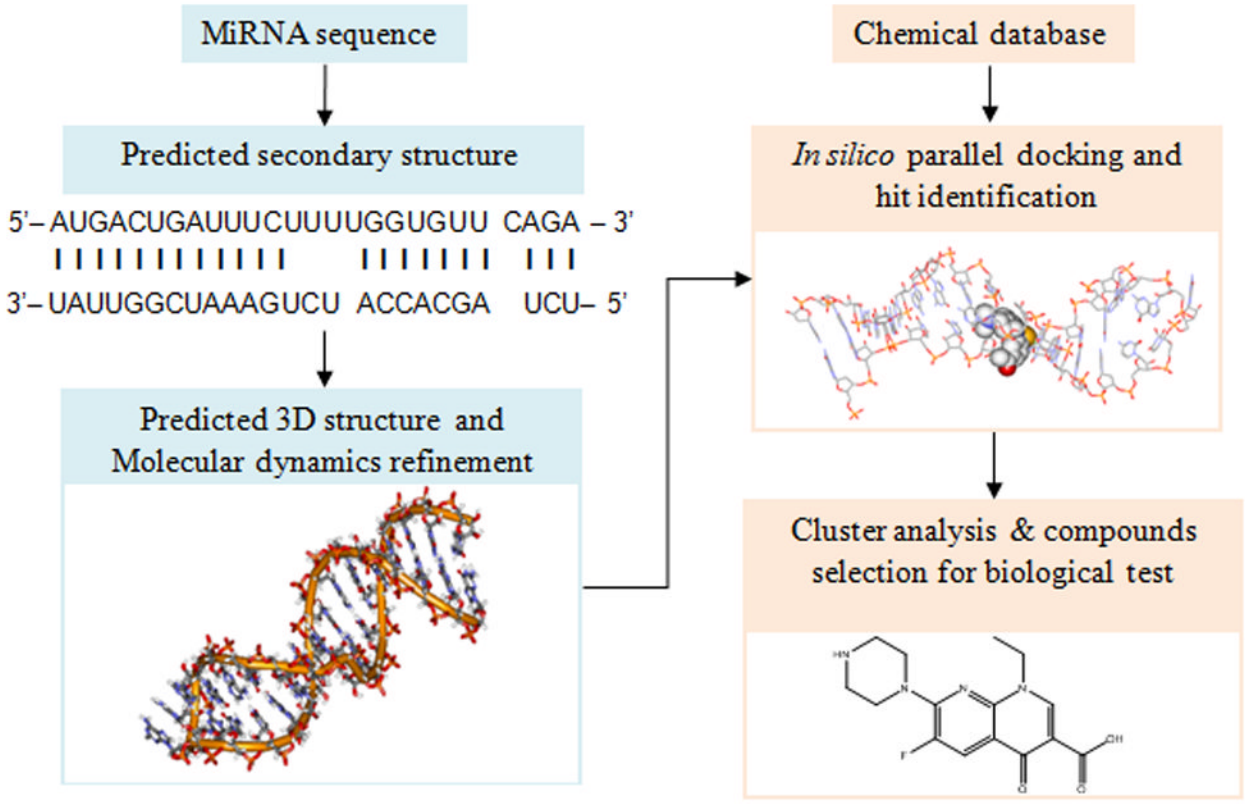


**Figure 1.**
*In silico* discovery of small molecule inhibitors targeting miRNAs as anticancer therapeutics.

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**TABLE 1**

Examples of oncogenic microRNAs in human cancers.

| Human microRNA | Deregulation in Tumors | Molecular mechanisms and Targets | Proved Targets * | Diagnostic and prognostic markers |
|---|---|---|---|---|
| *miR-17/18a/19a/20a/92 cluster* (13q31.3, intron 3 C13orf25) | • Overexpression in lung and colon cancers, and lymphomas, multiple myeloma, medullobalstoma | *Molecular mechanism:*<br>• *miR-17, -18a, -19a, -20a* and *-19b-1* accelerate tumor growth and increase tumor vascularization;<br>• *miR-20a* has an anti-apoptotic role;<br>• lymphoproliferative disease and autoimmunity in transgenic miR-17/92 cluster mice with increased expression in lymphocytes. | AIB1 AML1, BIM1, CTGF, CDKN1A, E2F1, E2F2, E2F3, HIF-1A PTEN, TGFBR2, TSP1, Rb2/P130. | *Diagnosis:*<br>• Plasma high levels discriminate CRC patients from normal and gastric cancer patients |
| *miR-21* (17q23.1, 3′UTR TMEM49) | • Overexpression in glioblastomas, breast, lung, prostate, colon, stomach, esophageal and cervical carcinomas, uterine leiomyosarcoma, DLBCL, and head and neck. | *Molecular mechanism:*<br>• *miR-21* knockdown induces apoptosis in glioblastoma cells;<br>• *miR-21* induces invasion and metastasis in colorectal cancers. | BCL2, MASPIN, PDCD4, PTEN, TPM1, RECK. RASA1 | *Poor prognosis:*<br>• *miR-21* high expression (in colon and breast cancer and pancreatic cancer).<br>*Good prognosis:*<br>• *miR-21* high expression in de novo DLBCL.<br>*Drug Resistance:*<br>• *miR-21* affects chemotherapy potency in NCI60 cells. |
| *miR-155* (21q21.3, exon 3 ncRNA BIC) | • Overexpression in pediatric BL, Hodgkin's disease, primary mediastinal lymphomas and DLBCL and in breast, lung, colon, and pancreatic cancers. | *Molecular mechanism:*<br>• Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in *miR-155* transgenic mice. | AGTR1, AID, IKBKE, TP53INP1. | *Poor prognosis:*<br>• *miR-155* high expression (in lung cancer, DLCBL, and aggressive CLL). |

* Target names as in NCBI database at http://www.ncbi.nlm.nih.gov/gene/; CRC - colorectal cancer; DLBCL - diffuse large cell B cell lymphoma; CLL -chronic lymphocytic leukemia.

**TABLE 2**

The main characteristics of the principal types of inhibitory RNAs drugs.

| Inhibitory RNA drugs | Definition | Mechanism of action | Drug development phase |
|---|---|---|---|
| Small molecules targeting miRNAs (SMIR) | Small synthetic organic molecules which can directly bind to miRNAs. Their molecule weight usually is less than 800 Da with ideal properties as drugs, including good solubility, bioavailability, PK/PD, metabolism, etc. | SMIRs bind to the grooves and pockets on the surface of miRNAs and interact with them directly, and thus interfere with biological functions of targeted miRNAs. | None |
| ANTISENSE OLIGONUCLEOTIDES (ASOs) | An antisense oligonucleotide is a single-stranded, chemically modified DNA-like molecule that is 17 to 22 nt in length and designed to be complementary to a selected messenger RNA and thereby specifically inhibit expression of that gene. | Formation of an mRNA-ASO duplex through Watson-Crick binding, leading to RNAse-H mediated cleavage of the mRNA of target gene. The ASOs also inhibit transcription, inhibit splicing and mRNA maturation, as well as ribosomal readthrough. | Clinical trials phase II and III |
| AMOs, LNAs anti-miR and antagomirs * | The AMOs are single-stranded, chemically modified DNA-like molecule that is 17 to 22 nt in length and designed to be complementary to a selected microRNA and thereby specifically inhibit expression of that gene. The LNAs anti miRNAs represents LNA modified ASOs.<br>The antagomirs are single-stranded 23-nucleotide RNA molecules complementary to the targeted microRNA that have been modified to increase the stability of the RNA and protect it from degradation. The modifications included a partial phosphorothioate backbone (PS) in addition to 2′-*O*-methoxyethyl. | AMOs are ASOs against miRNAs, and therefore produce ASO - miRNA duplex through Watson-Crick binding, leading to RNAse-H mediated cleavage of the target miRNA. The LNA anti miRNA have the same mechanism as the ASO/AMO<br>The antagomirs silences miRNA in a way still not completely known; the miRNA/antagomir – duplexes induces degradation of the miRNA and recycling of the antagomir. | Preclinical studies |
| RIBOZYMES or DNAZYMES | A ribozyme, or RNA enzyme, is an RNA molecule that can catalyze a chemical reaction. A DNAzyme, or dezoxyribozyme, is a catalytic DNA that site specifically cleaving the target RNA. | Watson-Crick base pairing to a complementary target sequence, then site-specific cleavage of the substrate and finally release of the cleavage products. | Clinical trials phase I and II |
| siRNAs | A siRNA is a double strand (ds) RNA homologous to an mRNA of a target gene. | The siRNAs are incorporated into a multiprotein RNA-induced silencing complex (RISC), leaving the antisense strand to guide this complex to its homologous mRNA target for endonucleolitic cleavage of messenger RNA. | Clinical trials phase I |

* AMOs - antisense microRNAs; LNA - locked nucleic acids.