Developing more specific miRNA biogenesis inhibitors to get effective regulation of miRNAs

Umesh Bhattarai
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DEVELOPING MORE SPECIFIC MIRNA BIOGENESIS
INHIBITORS TO GET EFFECTIVE REGULATION OF MIRNAS

by

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B.SC. CHEMISTRY, TRIBHUVAUN UNIVERSITY, 2007
M.SC. CHEMISTRY, TRIBHUVAUN UNIVERSITY, 2012

DISSEERTATION
Submitted in Partial Fulfillment of the
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Developing more specific miRNA biogenesis inhibitors to get effective regulation of miRNAs

by

Umesh Bhattarai

M.Sc. Chemistry, Tribhuvan University, 2012
PhD Chemistry, University of New Mexico, 2018

Abstract

MicroRNAs (miRNAs) are short (~22 nucleotides long) noncoding RNAs that regulate gene expression post-transcriptionally. They control the expression of various genes that are crucial for cellular function, development and human diseases. Tools to regulate the level and function of miRNAs facilitate the studies of their functions and have therapeutic applications. In my thesis, I developed a new customizable miRNA biogenesis inhibitor to regulate miRNAs. Antisense oligonucleotides (ASOs) have been used to regulate miRNA through controlling their production or function. Although commonly used due to the fact
that they are easy to design, several limitations exist for ASOs including serious off target effects, low cellular permeability and poor pharmacokinetics and distribution. To address these limitations, we proposed a new class of ASO based bi-functional molecules using short ASOs as the pre-miRNA recognition unit linked to a RNase III inhibitor as a Dicer inhibitor to block miRNA maturation. We showed the feasibility of the bi-functional strategy and optimized the bi-functional molecules through enhancing each functional unit as compounds 12, 22 and 25. Additionally, we identified cell penetrating peptide (CPP) that facilitate the delivery of bi-functional molecules to address the cell permeability issue.

We also explored the feasibility of using cyclic peptidomimetics as an alternative class of molecule to target RNAs, which can be used as our pre-miRNA recognition unit in the bi-functional regulator. We identified a new pre-miR-155 binder (compound 27) that inhibits miR-155 biogenesis. Applying RNAseq and bioinformatics analysis, we investigated its targeting selectivity and downstream effects.
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<th>Description</th>
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<tbody>
<tr>
<td>Pre-miRNA</td>
<td>Precursor microRNA</td>
</tr>
<tr>
<td>miR</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single stranded RNA</td>
</tr>
<tr>
<td>DGCR8</td>
<td>DiGeorge syndrome critical region 8</td>
</tr>
<tr>
<td>TRBP</td>
<td>TAR RNA-binding protein 2</td>
</tr>
<tr>
<td>AGO2</td>
<td>Argonaute 2</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA induced silencing complex</td>
</tr>
<tr>
<td>3'-UTR</td>
<td>3’-untranslated region</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>EC50</td>
<td>Half effective concentration</td>
</tr>
<tr>
<td>IC50</td>
<td>Half inhibitory concentration</td>
</tr>
<tr>
<td>ASO</td>
<td>Antisense oligonucleotide</td>
</tr>
<tr>
<td>MO</td>
<td>Morpholino</td>
</tr>
<tr>
<td>PNA</td>
<td>Peptide nucleic acid</td>
</tr>
<tr>
<td>LNA</td>
<td>Locked nucleic acid</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>25-MO-21</td>
<td>25-mer Morpholino ASO for pre-miR-21</td>
</tr>
<tr>
<td>25-MO-(C)</td>
<td>Negative control 25-mer MO</td>
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<td>16-MO-21</td>
<td>16-mer Morpholino ASO for pre-miR-21</td>
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<td>14-MO-21</td>
<td>14-mer Morpholino ASO for pre-miR-21</td>
</tr>
<tr>
<td>12-MO-21</td>
<td>12-mer Morpholino ASO for pre-miR-21</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>---------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>10-Mo-21</td>
<td>10-mer Morpholino ASO for pre-miR-21</td>
</tr>
<tr>
<td>25-MO-29b1</td>
<td>25-mer Morpholino ASO for pre-miR-29b1</td>
</tr>
<tr>
<td>14-MO-29b1</td>
<td>14-mer Morpholino ASO for pre-miR-29b1</td>
</tr>
<tr>
<td>T_m</td>
<td>Melting temperature</td>
</tr>
<tr>
<td>DMNB</td>
<td>4,5-dimethoxy-2-nitrobenzyl</td>
</tr>
<tr>
<td>DEACM</td>
<td>7-diethylaminocoumarin-4-yl methyl</td>
</tr>
<tr>
<td>CPP</td>
<td>Cell penetrating peptide</td>
</tr>
<tr>
<td>Anti-miR-155</td>
<td>MiR-155-5p targeting ASO</td>
</tr>
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</table>
Chapter 1

Introduction

1.1 Biogenesis and regulatory mechanism of miRNAs

Since the first discovery of microRNA (lin4) in 1993 which targets lin-14 gene and controls the larval development of C. elegans¹, more than 15,000 microRNAs (miRNAs or miR) have been discovered.² Out of these, more than 2500 are expressed in human cells and each of these is found to regulate the expression of numerous protein-coding genes (tens to hundreds). MiRNAs are ~ 21 to 23 nucleotide noncoding single-stranded RNAs (ssRNAs) that have important role in post transcriptional gene regulation either by mediating transcriptional repression or by degrading their target mRNAs. Furthermore, these non-coding RNAs play crucial roles in diverse biological activities such as cell proliferation, differentiation, metabolism as well as apoptosis³.

There are two pathways for the processing of miRNA genes transcribed from RNA polymerase II promoters, i.e. canonical and non-canonical pathways. In canonical pathway, RNA polymerase II transcribes miRNAs as primary miRNA (pri-miRNA) hairpins, which are further processed by the Drosha-DGCR8 (DiGeorge syndrome critical region 8) complex to precursor miRNAs (pre-miRNAs).⁴-⁶ Pre-miRNAs in turn transported into the cytoplasm by Exportin 5 and further processed by Dicer-TRBP (TAR RNA-binding protein 2) and loaded into Argonaute 2 (AGO2) to form RNA-induced silencing complexes (RISCs) to suppress downstream gene expression. In non-canonical pathway, pre-miRNAs are derived as the
spliceosome-dependent mechanisms bypassing Drosha and the formed pre-miRNAs are processed by Dicer to give mature miRNAs to be loaded into an RNA-induced silencing complex (RISC) as in the canonical pathway.\textsuperscript{7,8} RISC loaded

Figure 1.1: miRNA biogenesis and functions\textsuperscript{3}
with mature miRNA targets complementary 3'-UTR region of mRNA sequence and results in the post transcriptional gene silencing either by mRNA degradation or by translational repression or both, first translational repression followed by mRNA degradation\textsuperscript{9-12}. (Fig 1.1)

Primarily miRNAs act as post transcriptional gene regulator by targeting mRNA through base pairing, resulting in either mRNA degradation or translation repression depending on the extent of complementarity. The mature miRNA strand loaded within RISC complex, comprises a seed region of miRNA where nucleotide 2-8 precisely match the complementary sequence of 3'-UTR the mRNA to be targeted. Depending on the extent of base pairing outside the seed region, mRNA may either undergo degradation or translational repression. For example, perfect complementarity between miRNA and mRNA (mainly in plant cell) results into endonuclease mRNA degradation\textsuperscript{13} whereas imperfect complementarity with

![Figure 1.2: Mechanism of miRNA targeting.\textsuperscript{13}](image)

Figure 1.2: Mechanism of miRNA targeting.\textsuperscript{13}
some bulges or mismatched regions (mainly in animal cells) results in translational repression or followed by mRNA degradation\textsuperscript{14-17} (Fig 1.2).

Imperfect complementarity between miRNAs and targeted mRNAs can have direct effects on protein translation by causing the inhibitory action at the various stages of translation, e.g. repression at the initiation step by blocking the recruitment of 40S and/or 60S ribosomes near the 5’-cap of mRNA, ribosomal drop-off from mRNA by dissociation 40S/60S ribosomes, by hindering elongation through blocking ribosomes from joining during elongation process. Additionally, deadenylation of 3’poly-A tail of the mRNA will cause indirect translational repression resulting in the enhanced mRNA degradation (Fig 1.3).\textsuperscript{18-31}

![Diagram of translational repression](image)

Figure 1.3: Post-transcriptional gene regulation by miRNAs\textsuperscript{13}
1.2 Functions of miRNAs and their links to human diseases

MiRNAs play important roles in physiological homeostasis and pathophysiological conditions in diseases. Some miRNAs are more tissue specific and its expression level indicates certain pathological or physiological condition of related organs. Many researchers have found involvement of miRNAs in cell and tissue development, cancers, cardiovascular system, metabolism, viral infections, neuropsychiatric disorders etc. (examples in Table 1.1)\textsuperscript{3,13,32-54}

<table>
<thead>
<tr>
<th>Involved in</th>
<th>Role</th>
<th>Examples of miRNAs involved</th>
</tr>
</thead>
</table>
| Cell and Tissue Development         | • Proliferation and differentiation of embryonic stem (ES) cells.  
                                         • Lineage commitment during embryogenesis.  
                                         • Maturation of multiple tissues. | • miR-290 cluster and miR-21  
                                         • miR-133b  
                                         • miR-1, miR-133  
                                         • miR-143 |
| Cancer                              | • Tumorigenesis and Metastasis  
                                         • Oncogene suppressor          | • miR-17-92 cluster (oncomiR-1) and miR-10b  
                                         • miR-15a, miR-16-1, miR-126, |
<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
</table>
| **Cardiovascular system** | • Endothelial cell (EC) proliferation and angiogenesis  
  • Cardiac function  
  • Oxidative stress regulation | • Let-7f and miR-27b  
  • miR-1 and miR-133  
  • miR-21, miR-221 and miR-222 |
| **Metabolism** | • Tissue differentiation (involved in energy production, utilization and storage)  
  • Regulation of insulin release and metabolism  
  • Amino acid and lipid metabolism | • miR-375 and miR-29b  
  • miR-122, miR-103 and miR-107 |

Table 1.1: miRNAs in health and diseases

This understanding of cellular mechanisms of regulatory miRNAs and connections of their dysregulation with so many pathophysiological conditions suggest that possibility of exploiting miRNAs and their regulator stakeholders as a target to address and cure multiple pathophysiological conditions or diseases.
1.3 Current methods to regulate miRNAs

Several approaches have been developed to regulate the level or activity of miRNAs is based upon level and activity of mature miRNA, include using miRNA sponges as an alternative for miRNA targets or using small molecules or chemically modified antisense oligonucleotides as inhibitors.

**miRNA sponge**

This technique is based upon the overexpression of a transcript containing several artificial miRNA binding sites, each of which acts as binding site for endogenous miRNAs and altogether as sponges. As a result, the expression of target mRNA remains unaffected. Although this method is useful to investigate the miRNAs function *in vitro*, its dependency on cellular machinery to overexpress miRNA target sites and the requirement of special delivery methods for cellular uptake, makes it difficult for *in vivo* studies.\(^\text{55-58}\)

**Small molecule inhibitors**

Several small molecule-based miRNA inhibitors have been discovered and developed to control miRNA expression and function. Small molecules act mainly through regulation on the expression and maturation of targeted miRNAs. Aminoglycosides have long been regarded as good RNA binders, several of these aminoglycosides and modified aminoglycosides are reported as miRNA biogenesis inhibitors.\(^\text{59,60}\) Xanthone derivatives and other small molecules incorporating some aromatic and heteroaromatic moieties obtained by screening a library of small molecules against miRNA precursors have also been reported as
efficient miRNA biogenesis as well as functional inhibitors.\textsuperscript{61-66} Aza-flavones are also found to be an important class of miRNA inhibitors.\textsuperscript{67} Although some of the small molecule inhibitors are found to be effective in regulating miRNA function or expression, the relatively high EC50 value, the difficulty to design inhibitors based on target RNA sequences, and the limited understanding about the molecular mechanisms of inhibition by these molecules pose great challenges in therapeutic applications of this method.\textsuperscript{59-67}

**Antisense oligonucleotides**

Antisense oligonucleotide (ASO) technology has been the most commonly used method for miRNA regulation. One major advantage of using ASOs as oppose to small molecules is that the sequence-based design of inhibitors can be easily achieved. Most ASOs are designed to target miRNAs (e.g. anti-miRs) that inhibit the miRNA function or production by steric disruption of the interaction between miRNAs or pre-miRNAs and their protein partners (e.g. RNA-induced silencing complex (RISC) or Dicer), which lead to the inhibition of either their binding to endogenous mRNA targets or the maturation of miRNAs. Several chemically modified ASOs have been developed as anti-miRs to enhance their stability and binding affinity. Modifications like 2’-OMe are resistance to endonuclease, however, susceptible to degradation by exonuclease, which limits its applications. Phosphorothioate modification converting a non-bridging oxygen atom into sulfur offers an excellent exonuclease resistance and better cellular uptake. Mixed oligonucleotides with both 2’-OMe and phosphorothioate modifications have been used commonly as anti-miRs. Additionally, modifications like 2’-fluoro (2’-F), 2’-
methoxyethyl (2’-MOE) and locked nucleic acid (LNA) are also used to improve nuclease resistance while maintaining strong DNA/RNA binding affinity (Fig 1.5). These modifications can be used alone or mixed to tune the efficiency of anti-miRs. Backbone modifications (MO, PNA), although being used widely, these ASOs
typically have problem with cellular delivery, special methods are required to deliver these ASOs into cells.\textsuperscript{3}

![Chemically modified nucleotides](image)

Figure 1.5: Chemically modified nucleotides\textsuperscript{83}

Most of these ASOs act through competing with strong macromolecular interaction between miRNAs and their binding proteins. To offer effective competition, long ASOs are typically used (e.g. 25 nucleotides in case of MOs). Despite of consistency and efficacy, using long ASOs usually lead to off-target binding to other miRNAs, mRNA and other cellular RNAs.

Although with longer ASOs, we supposed to have fewer perfectly matched targets in the transcriptome, under physiological conditions, each ASO can interact with any RNAs through imperfect base pairing similar to non-specific priming observed in PCR at low annealing temperatures. As a result, small interfering RNA (siRNA) and miRNA mimetic including LNA and antagonirs have been shown to give
unwanted off-target effects.\textsuperscript{68-82} Such imperfect pairing is used by miRNA to regulate up to hundreds of mRNAs in the cells. Besides off-target inhibition, current ASOs cannot be used to dissect differential functions among miRNAs of same family (with similar mature sequence), along with the poor cellular delivery and distribution limit the utility of this strategy.

To address the issues of current ASO methods, my thesis is focused on the design and development of a new class of bi-functional miRNA regulators with distinct chemical structures and inhibitory mechanisms.
1.4 References


61) Murata, A., Fukuzumi, T., Umemoto, S. & Nakatani, K. Xanthone derivatives as potential inhibitors of miRNA processing by human Dicer:


Chapter 2

Developing ASO-based bi-functional miRNA regulators

2.1 Introduction

We designed a unique class of bi-functional miRNA regulators that blocks the Dicer-mediated maturation of miRNAs. Since Dicer knockdown causes serious biological defects including cell toxicity,\textsuperscript{4,5} the bi-functional molecules were designed to block the Dicer activity by selectively inhibiting the catalytic turnover of unique Dicer-pre-miRNA complexes to block the maturation of miRNA of interest. Dicer is a member of the ribonuclease III family that processes stem-loop double stranded pre-miRNAs (~ 70 nts) into mature miRNAs.

Figure 2.1: Downregulation of a specific miRNA through inhibiting its pre-miRNA cleavage by Dicer. (A bi-functional miRNA regulator selectively binds to the loop sequence of targeted pre-miRNA (red) and is brought to the associated Dicer. The inhibitor module in the regulator blocks the pre-miRNA cleavage by Dicer only at the targeted pre-miRNA.)
The designed bi-functional miRNA regulators contain a low affinity Dicer inhibitor covalently linked to a high affinity sequence-specific pre-miRNA recognition molecule to recognize and inhibit only specific Dicer molecules that would catalyze the cleavage of a specific pre-miRNA. At low concentrations, the weak binding Dicer inhibitor cannot inhibit Dicer activity on its own so that Dicer cellular function will remain intact. When bi-functional regulators bind to the loop region of targeted pre-miRNAs, the Dicer inhibitor will be placed at the active site of specific Dicer, with a dramatically increased local effective concentration of the Dicer inhibitor to achieve Dicer inhibition. There are many reported RNase III inhibitors that can potentially be used as our low affinity Dicer inhibitor through a two divalent metal ion catalyzed mechanism\textsuperscript{1-3} with IC\textsubscript{50} ranging from low to high µM concentration.\textsuperscript{6,7} Commercial custom synthesized morpholino oligonucleotides can were used as the high affinity pre-miRNA recognition module. MOs have standard nucleic acid bases linked to a morpholine rings connected through uncharged phosphorodiamidates instead of phosphodiester linkages (Figure 2.2), which gives the better binding affinity to RNAs as well as better nuclease resistance\textsuperscript{8-13}. As oppose to the standard long MOs (25-mer/ commonly used, we incorporated a short version of MOs 14-mer or less) to target the pre-miRNA loop region. We hypothesis that individual Dicer inhibitor unit and the pre-miRNA recognition unit by itself would not be inhibitory unless being coupled together to form the bifunctional regulator.
The function of miRNAs is usually dynamic and cellular context dependent. To achieve spatiotemporal regulation of miRNAs, we also developed light controllable bi-functional miRNA regulators. ASOs such as morpholinos have poor cell permeability and enhanced stability in cells. They cannot get in the cell readily, and once delivered into the cells, the inhibitory effect of ASOs can be long lasting. To offer a spatiotemporally inactivatable miRNA regulator, we designed a light deactivatable version of bi-functional regulator by connecting the pre-miRNA recognition unit with the Dicer inhibition unit through a photo-cleavable linker. Once cleaved by light the bifunctional molecule will be fragmented into two inactive units and the inhibitory effects reversed.

![Diagram of light controllable bifunctional molecules](image)

**Figure 2.3: Design of light controllable bifunctional molecules**

Furthermore, a light activatable bi-functional regulator can be generated by masking the Dicer inhibition unit with a photo-cleavable caging group. Such regulators will remain inactive until the caging group is removed by a specific
radiation of light to release the active bi-functional miRNA regulator. By combining both of these light activation and inactivation strategies, a bi-functional miRNA regulator with precise spatiotemporal control can be created (Figure 2.3).

2.2 Results and discussions

Recognition module

To start our investigation about the bi-functional strategy, we choose to use MO as our pre-miRNA recognition unit, which is designed to be complementary to the single stranded hairpin loop region of pre-miRNA of interest. This will facilitate the targeting of exposed sequences and avoid the binding competition with Dicer at the Dicer cleavage site in the double stranded region. 25-mer MOs (T_m ~ 90°C)\(^1\)\(^4\) are currently used to provide the desired knockdown effects, which cause off target inhibitions under physiological condition likely due to imperfect pairing to many RNA species in cells. To address this, we used shorter 14-mer MOs (T_m ~60°C)\(^1\)\(^4\) as the pre-miRNA recognition unit, which we expect to have reduced off targets and still form sufficiently stable duplex with pre-miRNAs in cells. Since MOs longer than 15-mer have been found to have observable biological effects, 14-mer MO (or shorter) should not block Dicer-mediated pre-miRNA processing by itself.

To investigate if a 14-mer MO is sufficient to specifically match a unique pre-miRNA in sequence, we performed a BLAST analysis using 14-mer sequences chosen from the hairpin-loop region of miRNAs associated with human diseases. Among all the tested sequences, each only matches one unique pre-miRNA. Although many of these sequences also exist in mRNAs (Figure 2.4), our unique
bi-functional design, which exerts effects through Dicer enzymatic inactivation instead of steric interference, minimizes the interference on mRNAs.

We have chosen miRNA-21 as our initial target for the study, which was first identified in 2005 as the most commonly and highly upregulated miRNA in human brain tumor glioblastoma\textsuperscript{15}. Since then many researchers studied about this miR-21 in various fields including oncology, stem cell and aging biology and development, making it one of the most studied miRNAs (Table 2.1).

![NCBI blast analysis of pre-miR loop sequence](image)

Figure 2.4: BLAST analysis of 14-mer pre-miRNAs loop sequences

<table>
<thead>
<tr>
<th>Disease cells</th>
<th>miR-21 expression</th>
<th>Biological process</th>
<th>miR-21 targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glioblastoma</td>
<td>Up-regulation</td>
<td>Invasion and cell growth</td>
<td>PDCD4, RECK,</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>miRNA</th>
<th>mRNA</th>
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<tr>
<td>miRNA-21</td>
<td>miRNA-208a</td>
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<tr>
<td>miRNA-15a</td>
<td>miRNA-17</td>
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<tr>
<td>miRNA-15b</td>
<td>miRNA-15a</td>
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<td>let-7a-1</td>
<td>miRNA-15b</td>
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<tr>
<td>miRNA-16-1</td>
<td>miRNA-93a</td>
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<tr>
<td>miRNA-221</td>
<td>miRNA-107</td>
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<tr>
<td>miRNA-26a-1</td>
<td>miRNA-34a</td>
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<td>miRNA-9-1</td>
<td>miRNA-205</td>
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<td>miRNA-206</td>
<td>miRNA-200a</td>
</tr>
<tr>
<td>Cancer Type</td>
<td>Regulation</td>
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<tr>
<td>-----------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>Up-regulation</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>Up-regulation</td>
</tr>
<tr>
<td>Pancreas cancer</td>
<td>Up-regulation</td>
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<tr>
<td>Ovarian cancer</td>
<td>Up-regulation</td>
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<tr>
<td>Cervical cancer</td>
<td>Up-regulation</td>
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<td>Colorectal cancer</td>
<td>Up-regulation</td>
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<tr>
<td>Hepatocellular carcinoma</td>
<td>Up-regulation</td>
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<tr>
<td>Stomach/gastric cancer</td>
<td>Up-regulation</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>Up-regulation</td>
</tr>
<tr>
<td>Thyroid carcinoma</td>
<td>Up-regulation</td>
</tr>
</tbody>
</table>
Leukemia | Up-regulation |
---|---|
B-cell and Hodgkin lymphoma | Up-regulation |
Cardiovascular | Up/down regulation |

Vascular smooth muscle cell proliferation and apoptosis, cardiac cell growth and death, and cardiac fibroblast functions | PDCD4, PTEN, SPRY1, SPRY2 |

Table 2.1: miR-21 regulation and function in human diseases^{15-21}

<table>
<thead>
<tr>
<th>Hair-pin secondary structure of hsa-pre-miR-21^{22-25}</th>
<th>(yellow highlighted region is the sequence we want to target through our recognition module i.e. MO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'</td>
<td>gu a a a a u a</td>
</tr>
<tr>
<td>5'</td>
<td>ugucgg agcuuauc gacug uguug cugu g a</td>
</tr>
<tr>
<td>3'</td>
<td>acaguc ucggguag cugac acaac ggua c c</td>
</tr>
<tr>
<td>3'</td>
<td>ug - c - - u</td>
</tr>
</tbody>
</table>

Based upon the sequence of hsa-premiR-21 (Figure 2.5), we have designed the following MOs with a 3’-azido group, which can be custom synthesized (GeneTools, Philomath, OR), to be used as our pre-miR-21 recognition unit,

5’-CCATGAGATTCAACAG-3’ (16-mer MO-21)
5’-CCATGAGATTCAACA-3’ (15-mer MO-21)
5’-CATGAGATTCAACA-3’ (14-mer MO-21)
Furthermore, we used a 25-mer MO-21 (as below) designed by GeneTools as miR-21 inhibitor, which is complementary to both mature strand sequence and part of the loop region as a positive control.

AGTCAACATCAGTCTGATAAGCTAC (25-mer MO-21)

**Comparison of off-target binding of 14-mer versus 25-mer MO against cellular RNAs in vitro**

We expect that under physiological temperature, a 25-mer MO will likely target a lot more different mRNAs through imperfect pairing than a 14-mer, which is a potential source of causing unwanted biological effects.

To test if a 14-mer MO indeed recognize less RNAs in the transcriptome, we got biotin-labeled 14-mer and 25-mer MOs for recognizing pre-miR-21 by click chemistry between the azido-MOs and an alkyne-group attached biotin. These MOs were incubated separately with total mRNAs purified (using Oligotex mRNAs kit from QIAGEN, Hilden, Germany) from HeLa cells (known to express miR-21) at 37°C and the bound RNA species were pull-downed by streptavidin beads. We
had also incubated biotin only (without MO) with these RNAs as a negative control. After washing, the bound RNAs with beads were eluted and submitted for RNA-seq (using Illumina NextSeq 500 for pair-end sequencing 75bp X 2, 130 million read). As an indication of the number of RNAs each molecule target, we focused on mRNAs being pulled down in each condition. Obtained reads were aligned against human reference genome, annotated and counted. After removing common RNAs found in biotin-only pull down, we found that the 14-mer MO indeed bound much less mRNAs than 25-mer MO (Figure 2.6).

**Inhibitor and bifunctional molecules**

To generate a bi-functional regulator, we chose to use reported N-substituted Phthalimides (-OH, -OMe, -NH₂) (e.g. compound 1, 2 and 3, Figure 2.7) as our Dicer inhibition unit, which have been reported to inhibit RNase III family enzymes by chelating two divalent metal ions (Mg²⁺ in Dicer) in the active site, although rather poorly (IC₅₀ > 500 µM).⁶ Furthermore, some other more potent RNase III inhibitors (low µM) e.g. N-hydroxyimides, diketobutanoates or flutimides (compound 4, 5 and 6, Figure 2.7)⁶,⁷ can also be used.

![Chemical structures](image)

**Figure 2.7: Some examples of RNase III inhibitors**⁶,⁷.
We expect that the length and the structure of linkers may also play a crucial role in the bi-functional molecules activity in addition to the Dicer inhibition unit and the pre-miRNA recognition unit. As a result, we synthesized and tested several bi-functional miR-21 regulators with different linkers to connect the two functional units.

Scheme 2.1: Synthesis of bi-functional miRNA regulators with different linker length units.

To synthesize bi-functional regulators, we synthesized alkyne-equipped N-substituted phthalimides (-OH, -OMe, -NH₂) (compound 1, 2 and 3) that were subsequently linked to azido-MOs custom synthesized for pre-miR-21 through copper catalyzed click chemistry²⁶ (compound 10 to 13) (Scheme 2.1). To test the importance of the distance between the Dicer inhibition unit and the pre-miRNA
recognition unit, we synthesized another bi-functional molecule with longer linker (21 atoms vs. 10 atoms) to connect the two functional units as shown in Scheme 2.1 (Compound 14).

All synthesized molecules were purified by HPLC or silica gel chromatography and fully characterized before biological assays to ensure the purity (without the contamination of other organic solvents or reagents that were used during synthesis). Using click chemistry to assemble the bi-functional molecules will enable us to generate the new bi-functional regulators for other miRNAs by switching the pre-miRNA recognition units with MOs designed for other pre-miRNA sequences.

**Stability of bifunctional molecules**

To test the stability of the bi-functional molecule, we incubated compound 12 in the HEPES buffer (pH 7.4, physiological condition) for up to 5 d and phosphate buffer (pH 6, endosome release condition\(^\text{27}\)) for up to 48 h and then analyzed by HPLC. We found that the synthesized bi-functional regulator is chemically stable during

Figure 2.8: Chemical stability of the bi-functional regulator by HPLC at different pH
tested period based on the finding that the peak corresponding to compound 12 in HPLC chromatogram did not change after the incubation (Figure 2.8).

To further explore the binding of our pre-miR-21 recognition units (i.e. MOs) with pre-miR-21, gel shift assay was performed. All the recognition units, the bifunctional molecule (12), or the positive control (25MO-miR-21) and negative control (25MO-miR-29b1) were incubated with P32-internally labelled premiR-21 at 37 °C for 1.5 h and then subjected to 15% non-denaturing polyacrylamide gel analysis, followed by exposure to photographic plate and imaging using PMI1 (Figure 2.9). Gel shift assays showed 14-mer MO (or longer), as well as the bifunctional molecule, stably bound to pre-miR-21 whereas 12-mer MO or 25-mer MO designed for another pre-miRNA did not bind pre-miR-21. These results confirm that the 14-mer MO is sufficient to recognize target pre-miRNA.

Dicer cleavage inhibition assay of bifunctional molecules

Our bi-functional miRNA regulators are designed to inhibit Dicer processing of pre-miRNA. To evaluate Dicer activity on pre-miRNA processing, we developed an in vitro assay that includes the recombinant human Dicer proteins (from Genlantis, San Diego, CA) and 32P-labeled pre-miRNA (produced by the in vitro transcription from DNA template with T7 promoter using T7 RNA polymerase). By using this method, we can successfully examine Dicer-mediated processing of pre-
miRNAs (e.g. pre-miR-21) (~70 nts) into short mature miRNA (~22 bps) using 16% denaturing polyacrylamide gel electrophoresis with Decade marker system (Thermo Fisher Scientific) followed by exposure to photographic plate and imaging using PMI (Figure 2.10).

We then used this assay to test the activity of bi-functional regulators, different MO, inhibition units and the control 25-mer MO against Dicer-mediate processing of pre-miR-21. We found that none of the recognition units significantly inhibit the processing (up to 100 µM, Figure 2.11). Similarly, none of the synthesized Dicer inhibition units (compound 7 to 9 up to 1mM) inhibit the processing. In this figure, 25 MO (C) is the scrambled negative control 25-mer MO designed by Gene-Tools (Figure 2.12). On the contrary, the bi-functional miR-21 regulator with 14-mer MO (compound 12) blocked the Dicer-mediated pre-miR-21 cleavage process at 10 µM and above, showing a potency improvement of at least 100 folds as compared to its corresponding inhibition unit (compound 7) (Figure 2.13). Interestingly, the bi-functional regulator with the longer flexible linker (compound 14), showed a much lower activity as compared to compound 12 (Figure 2.13).
Figure 2.11: Dicer cleavage assay for recognition units A) Gel electrophoresis of cleaved and un-cleaved pre-miR-21 B) quantification of pre-miR-21 band in each lane (3-independent experiments were used to calculate the error bar)

Figure 2.12: Dicer cleavage assay for inhibition units A) Gel electrophoresis of cleaved and un-cleaved pre-miR-21 B) quantification of pre-miR-21 band in each lane (3-independent experiments were used to calculate the error bar)
This suggests that the importance of linker in our bi-functional molecule and the decreased activity may due to the entropic penalty introduced by using a longer and flexible linker. Encouragingly, bi-functional regulator with the short linker (compound 12) showed a better activity than the 25-mer MO control at the similar concentration (Figure 2.13). Overall, we showed that the bi-functional design can give functional regulators to block miRNA maturation. The 14-mer MO can give sufficient recognition without inhibiting the process by itself. The linker plays an important role in the activity. To further explore, the effects of the MO length in the activity of bi-functional molecules, we synthesized bi-functional molecules with shorter or longer MOs range from 10-16-mer (Scheme 2.1) and tested their activity to block miRNA maturation in vitro.
From the results, we found that bi-functional regulators with MOs shorter than 14-mer (compound 10 and 11) have decreasing activity corresponding to the MO length (Figure 2.14A). Surprisingly, compound 13 (with 16-mer MO) has decreased activity when compared to compound 12 (with 14-mer MO) (Figure 2.13B). These results suggest that the pre-miRNA recognition unit plays a key role in the activity of the bi-functional regulator.

![Figure 2.14: Dicer cleavage assay for different length recognition unit bifunctional molecules A) and B) Gel electrophoresis of cleaved and un-cleaved pre-miR-21 C) and D) quantification of pre-miR-21 band in each lane (3 independent experiments were used to calculate the error bar) for A and B respectively](image_url)
Generating bi-functional regulators for other miRNAs

Based on the findings shown above, we hypothesize that new bi-functional regulators can be generated to target other miRNAs by swapping the pre-miRNA recognition unit. To test this, we synthesized bi-functional regulators for hsa-miR-29s by replacing the MO unit in the bi-functional miR-21 regulators with an MO designed to target the loop sequence of pre-miR-29b-1. There are four hsa-miR-29, namely hsa-miR-29a, hsa-miR-29b-1, hsa-miR29b-2 and hsa-miR-29c in the family. Out of four members, miR-29a, miR-29b1 and miR-29c are the most abundant and functionally important miRNAs.\textsuperscript{1,28-37}

hsa-pre-miR-29a
5'AUGACUGAUUUUCUUUGUGUUCAG\textcolor{yellow}{AGUCAAAUAUUUUCUAGCACCAUCUGAAAUUCGUAAU} 3'
hsa-pre-miR-29b-1
5'\textcolor{green}{CUUCAGGAAGCUGGUUUCUAAUGGUGGUUUAGAUUAAAUAGUGAUUGUCUAGCACCAUUUGAAAUCAGUGUUCUUGGGG} 3'
hsa-pre-miR-29c
5'AUCUCUUUACACAGGCUACCGAUUUCUCCUGGUGUUC\textcolor{pink}{AGAGUCUGUUUU} UUGUCUAGCACCAUUUGAAUCGGUUA\textcolor{green}{UGAUGUAGGGGGA} 3'

Figure 2.15: Nucleotide sequence of hsa-pre-miR-29 family (The yellow highlighted regions represent the loop-sequences, green regions represent the 3p-mature sequences and pink regions represent the less abundant 5p-mature sequences)
MiR-29 family members share almost identical mature sequences but each has unique loop sequence among others (Figure 2.16). It is known that miR-29a and miR-29b-1 are both expressed in Hela cells. However, miR-29a resides in cytoplasm and miR-29b-1 is transported to the nucleus. They also have different half-life during cell cycles. It suggests that miRNAs within the same family potentially have different functions.

Since the majority of miRNA genes (~ 70%) are reported to be grouped into miRNA families, it is very important to dissect the function of each member in a family. Current miRNA regulators cannot selectively target individual members within the same family because most of these techniques target the highly homologous mature miRNA sequence shared by miRNAs in the same family. Our bi-functional miRNA regulators targeting the unique loop sequences might be an efficient way to distinguish individual miRNAs in a family.

To develop a new regulator for miR-29b1 from the bi-functional miR-21 regulator, we designed our recognition unit (a 3’-azido14-mer MO) to be complementary to the loop region of pre-miR-29b-1. We also obtained the 25-mer MO antagonir from Gene tools as a control.8-12
5’-AACACTGATTCTAAATGGGTGCTAGA-3’  
(25-mer MO-29b1 antagonir)

5’-ACAATCATACTATTTA-3’  
(14-mer MO-29b1)

We synthesized the bi-functional regulator (compound 15) by clicking the azido 14-mer MO for miR-29b-1 and inhibition unit (compound 7) and tested its activity using the Dicer cleavage assay.

We observed that compound 15 can completely inhibit the processing of pre-miR-29b-1 by Dicer and is more potent than the 25-mer MO control at the same
concentration (Figure 2.17). These results indicate that new bi-functional miRNA regulators can be quickly generated by switching the pre-miRNA recognition units.

**Design and synthesis of light controllable bifunctional molecule**

To develop a light controllable version of bi-functional regulators to achieve spatiotemporal control, we incorporated a photo-cleavable 4, 5-dimethoxy-2-nitrobenzyl (DMNB) group (cleavable by 365nm light) in to the linker connecting the inhibition unit and the recognition unit (compound 16). This will allow a rapid light inactivation of the bi-functional molecules by separating the two functional units. We also synthesized the corresponding bi-functional molecule (compound 17) by clicking the azido 14-mer MO to compound 16. Furthermore, to achieve light activation, it is possible to attach a photo-cleavable [7-diethylaminocoumarin-4-yl]methyl (DEACM) group to the inhibitor unit at the N-hydroxy group (e.g. compound 18) that can be removed by the 405nm light.

Scheme 2.2: Synthesis of light-controllable bi-functional regulator
Test the light cleavage efficiency and inactivation of bi-functional molecule

To test the light induced cleavage, we illuminated the synthesized light-controllable molecule (compound 16) with 365 nm light for different time periods and did HPLC analysis. We found that the DMNB group on compound 16 can be efficiently cleaved by 365 nm light (Figure 2.18).

To test if the light irradiated DMNB-containing bi-functional regulator can be inactivated, we compared the inhibitory activity of compound 16 before and after 365nm light irradiation (10 min) using the Dicer cleavage assay. We observed that the activity of the light controllable bi-functional molecule (compound 16) was abolished after light irradiation (Figure 2.19).

![HPLC analysis cleavage efficiency of compound 16 by using 365 nm light](image)

Figure 2.18: HPLC analysis cleavage efficiency of compound 16 by using 365 nm light
Facilitating cellular delivery of bi-functional regulators

One of the main problems associated with antisense strategy is the cellular delivery. One common method used to deliver MO is the use of a delivery agent ‘Endo-porter’ (designed by Gene-tools).\textsuperscript{50-53} To test the cellular delivery efficiency, we synthesized a model bi-functional regulator with a 14-mer MO and a fluorescent probe (compound 19) (Scheme 2.3).

![Scheme 2.3: Synthesis of the fluorescent bi-functional MO probe.](image)

Figure 2.19: Dicer cleavage assay for active vs deactivated light controllable bi-functional molecule A) Gel electrophoresis of cleaved and un-cleaved pre-miR-21 B) quantification of pre-miR-21 band in each lane (3-independent experiments were used to calculate the error bar)
We tested the delivery of compound 19 (10 µM) into Hela cells using Endo-Porter following vendor’s protocol and observed the fluorescence signal under a fluorescence microscope in presence of Endo-porter. We did observe the delivery of the model molecule inside the cells although the level was very low and not very efficient (it took about 72h before a fluorescence signal can be observed in the cell) (Figure 2.20).

We then tested using Endo-porter to deliver the bi-functional miR-21 regulator (compound 12) into HeLa cells and examined the changes of the cellular miR-21 level with or without the treatment of the regulator. Unfortunately, no significant cellular activity was observed by RT-qPCR (monitoring miR-21 levels) and western blot analysis (monitoring PDCD4 levels, a miR-21 target). The lack of cellular activity is likely due to the ineffective delivery method, or insufficient activity. In the next part of my work, I am focusing on optimizing the bi-functional regulators, including the delivery methods.

Figure 2.20: Cell uptake of the fluorescent bi-functional MO probe (compound 19) in Hela cells (A= none, B= fluorophore compound only, C= compound 19 only and D= compound 19 + Endo-porter) after 72 hrs.
2.3 Conclusion

In this Chapter, we described the design and development of a unique class of bi-functional regulators. We showed that the bi-functional strategy improves the activity of individual Dicer inhibition and pre-miRNA recognition units. We identified compound 12 the most potent bi-functional molecule.

Furthermore, with the intention to develop spatiotemporally controlled miRNA regulation tools, we converted compound 12 into light controllable molecule by incorporating a light cleavable group in its linker to make it become light inactivatable (compound 17).

Additionally, the current delivery method, Endo-porter, is found to be inefficient to deliver our bi-functional molecules and new delivery methods are required.
2.4 Methods

Bifunctional molecules synthesis

Compound 2 was synthesis as described in the literature 1.

1-Hexynoic acid (1.2 g; 11.24 mmol) and BOP reagent (5.7 g; 13 mmol) were dissolved in CH2Cl2 (15 mL). After 10 min, 2, 2″-(Ethyleneedioxy) diethylamine (10 g; 67.47 mmol) in CH2Cl2 (60 mL) was added. The mixture was stirred at room temperature for 3 hours. A saturated NaHCO3 aqueous solution (pH 9, 50 mL) was added and organic layer was extracted several times by CH2Cl2 (50 mL). The organic phase was filtered, dried with Na2SO4 and evaporated under reduced pressure. The residue was purified by silica gel column chromatography.
(CH2Cl2/MeOH/Triethylamine, 95/5/0.1) to provide 2 g of (2) (yield 73 %) as a yellow oil. Rf: 0.31 (CH2Cl2/MeOH/Triethylamine, 9/1/0.1).

Compound 4 was made from compound 3 follow the routine methods.

Compound 5

Propargylamine (55 mg, 1 mmol) was dropped into a solution containing Compound 4 (211 mg, 1 mmol) in dry THF under ice bath. Followed by Et3N (121 mg, 1.2 mmol). Continue stir 3 hours. Remove the solvent under 30 °C. NH2NH2 (70 mg, 1 mmol) and pyridine (20 mL) were added to the flask and stirred 15 h at 90 °C. Then remove the solvent, wash with water and dry it. Yield: 68 %. 1H NMR (DMSO-d6, 300 MHz): 11.66 (bs, 2H), 9.39 (t, J= 10.8 Hz, 1H), 8.57 (s, 1H), 8.31-8.28 (dd, J= 8.4, 1.8 Hz, 1H), 8.14-8.11 (d, J= 8.1 Hz, 1H), 4.11-4.08 (q, J= 7.8 Hz, 2H), 3.17-3.15 (t, J= 4.8 Hz, 1H). 13C NMR (DMSO-d6, 75 MHz): 164.80, 137.13, 131.21, 128.76, 127.42, 125.54, 124.45, 80.98, 73.07, 28.80. TOF-HRMS (m/z) found (calcd.) for C12H9N3O3 (M): [M+H]+, 244.0721 (244.0722).

Compound 6

Propargylamine (55 mg, 1 mmol) was dropped into a solution containing Compound 4 (211 mg, 1 mmol) in dry THF under ice bath. Followed by Et3N (121 mg, 1.2 mmol). Continue stir 3 hours. Remove the solvent under 30 °C. NH2OCH3/HCl (84 mg, 1 mmol) and pyridine (20 mL) were added to the flask and stirred 15h at 90 °C. Then remove the solvent and purify on silica gel. Yield: 75 %. 1H NMR (DMSO-d6, 300 MHz): 9.34-9.30 (t, J= 10.8 Hz, 1H), 8.30-8.26 (m, 2H), 7.96-7.94 (dd, J= 7.8, 0.6 Hz, 1H), 4.11-4.09 (q, J= 7.8 Hz, 2H), 3.97 (s, 3H), 3.19-
3.18 (t, J= 4.8 Hz, 1H). 13C NMR (DMSO-d6, 75 MHz): 164.03, 162.37, 162.30, 139.09, 133.87, 130.95, 128.99, 123.43, 121.55, 80.74, 73.25, 56.42, 28.83. TOF-HRMS (m/z) found (calcd.) for C13H10N2O4 (M): [M+H]+, 259.0710 (259.0719); [2M+Na]+, 539.1189 (539.1179).

Compound 7

Propargylamine (55 mg, 1 mmol) was dropped into a solution containing Compound 4 (211 mg, 1 mmol) in dry THF under ice bath. Followed by Et3N (121 mg, 1.2 mmol). Continue stir 3 hours. Remove the solvent under 30 °C. NH2OH/HCl (70 mg, 1 mmol) and pyridine (20 mL) were added to the flask and stirred 15 h at 90 °C. Then remove the solvent and purify on silica gel. Yield:75 %.

1H NMR (DMSO-d6, 300 MHz): 10.93 (s, 1H), 9.34-9.30 (t, J= 10.8 Hz, 1H), 8.29-8.25 (m, 2H), 7.95-7.92 (d, J= 7.5 Hz, 1H), 4.11-4.08 (q, J= 7.8 Hz, 2H), 3.17-3.16 (t, J= 5.1 Hz, 1H). 13C NMR (DMSO-d6, 75 MHz): 164.15, 163.53, 163.46, 138.97, 133.65, 131.068, 129.11, 123.16, 121.31, 80.73, 73.21, 28.78. TOF-HRMS (m/z) found (calcd.) for C12H8N2O4 (M): [M+H]+, 245.0560 (245.0562).

Compound 8

Copper (I) iodide (50 nmol), Sodium Ascorbate (VcNa) (100 nmol) and Tris[(1-benzyl-1H-1,2,3-triazol-4-yl) methyl] amine (50 nmol) were mixed together in DMSO. Followed by Morpholinos (50 nmol) in water, small molecule (100 nmol) in DMSO. The mixture was shacked and incubated 15h. Separate on HPLC.

8a: MS (modify protein way) (m/z) found (calcd.): 3843.8 (3844.1).
8b: MS (modify protein way) (m/z) found (calcd.): 4529.9 (4529.1).

8c: MS (modify protein way) (m/z) found (calcd.): 5183.8. (5184.1).

8d: MS (modify protein way) (m/z) found (calcd.): 5501.5 (5501.1).

8e: MS (modify protein way) (m/z) found (calcd.): 5856.8 (5857.1).

Compound 9

Compound 9 was synthesized follow the method as described for compound 7. Yield: 35 %.

1H NMR (MeOD, 300 MHz): 8.27-8.25 (m, 2H), 7.95-7.92 (d, J= 7.8 Hz, 1H), 3.71-3.63 (m, 8H), 3.58-3.54 (t, J= 10.5 Hz, 2H), 3.37-3.33 (t, J= 12.9 Hz, 2H), 2.33-2.28 (t, J= 14.7 Hz, 2H), 2.24-2.18 (m, 3H), 1.83-1.74 (m, 2H). 13C NMR (MeOD, 75 MHz): 164.15, 163.53, 163.46, 138.97, 133.65, 131.068, 129.11, 123.16, 121.31, 80.73, 73.21, 28.78. TOF-HRMS (m/z) found (calcd.) for C21H25N3O7 (M): [M+Na]+, 454.1588 (454.1590).

Compound 10

Compound 10 was synthesized follow the method as described for compound 8.

MS (modify protein way) (m/z) found (calcd.): 5371.5 (5371.2).

**Synthesis for bi-functional molecule for miR-29-b-1**

Same as in method described for compound 8 but using 14 MO-29b1 with compound 7.

MS (modify protein way) (m/z) found (calcd.): 5134.96 (5,135.056).
Light cleavable bifunctional molecule synthesis

Compound 2 was made from compound 1 following the routine methods.

Compound 4

Compound 3 (1.69 g, 10 mmol) and K2CO3 (1.66 g, 12 mmol) were stirred in DMF 10 min at 90 °C. Propargyl bromide (80 wt. % in toluene, 12 mmol) was added to above solution. Continue stirring 2h. Extract with Ethyl acetate/ brine. Purify on silica gel. Yield: 98 %. 1H NMR (Acetone-\textit{d}6, 300 MHz): 8.18-8.15 (d, J = 9.0 Hz, 1H), 7.56-7.55 (d, J = 1.5 Hz, 1H), 7.11-7.07 (dd, J = 9.0, 2.4 Hz, 1H), 5.03 (d, J= 5.4 Hz, 2H), 4.97-4.96 (d, J= 2.1 Hz, 2H), 4.70-4.66 (t, J= 11.1Hz, 1H), 3.18-3.16 (t, J= 3.6 Hz, 1H). 13C NMR (Acetone-\textit{d}6, 75 MHz): 162.00, 142.14, 140.47, 127.20, 113.85, 113.02, 77.86, 76.99, 60.95, 56.06. TOF-HRMS (m/z) found (calcd.) for C10H9NO4 (M): [M+Na]^+, 230.0423 (230.0429).

Compound 5
Compound 4 (1.03 g, 5 mmol) was dropped into a solution containing Compound 4 (1.05 g, 5 mmol) in dry THF under ice bath. Followed by Et3N (5.56 g, 5.5 mmol). Continue stir 5 hours. Remove the solvent under 30 °C. NH2OH/HCl (0.35 g, 5 mmol) and pyridine (50 mL) were added to the flask and stirred 15 h at 90 °C. Then remove the solvent and purify on silica gel. Yield: 45%. 1H NMR (Acetone-d6, 300 MHz): 8.57-8.54 (d, J = 7.7 Hz, 1H), 8.41 (s, 1H), 8.29-8.26 (d, J = 9.0 Hz, 1H), 8.03-8.00 (dd, J= 7.8, 0.6 Hz, 1H), 7.47-7.46 (d, J= 2.7 Hz, 1H), 7.25-7.21 (dd, J= 9.0, 2.8 Hz, 1H), 5.85 (s, 2H), 5.00-4.99 (d, J= 2.4 Hz, 2H), 3.22-3.20 (t, J= 4.8 Hz, 1H). 13C NMR (Acetone-d6, 75 MHz): 163.99, 162.82, 161.86, 141.14, 135.59, 135.10, 134.66, 133.29, 129.94, 127.75, 123.36, 115.03, 114.48, 77.66, 77.44, 64.12, 56.28. TOF-HRMS (m/z) found (calcd.) for C19H12N2O8 (M): [M+Na]⁺, 419.0496 (419.0491).

Compound 7

Compound 5 (0.396 g, 1 mmol) and 1, 8-Diazabicyclo[5.4.0]undec-7-ene (DBU, 0.182 g, 1.2 mmol) stir 10 min in DCM. Compound 6 was added to above solution and stirred 2 more hours. After reaction is finish. Remove solvent, purify on silica gel. Yield: 80 %. 1H NMR (CDCl3, 300 MHz): 8.48-8.45 (dd, J = 7.8 Hz, 1H), 8.28 (s, 1H), 8.26-8.23 (d, J = 9.0 Hz, 1H), 8.06-8.03 (d, J= 7.8, Hz, 1H), 7.74-7.71 (d, J= 9.0 Hz, 1H), 7.37-7.36 (d, J= 2.7 Hz, 1H), 7.24-7.20 (dd, J= 9.2, 2.9 Hz, 1H), 6.72-6.69 (dd, J= 9.0, 5.4 Hz, 1H), 6.52 (d, J= 2.1 Hz, 1H), 6.27 (s, 1H), 5.75 (s, 2H), 5.35 (s, 2H), 5.00-4.99 (d, J= 2.4 Hz, 2H), 3.66-3.64 (t, J= 4.5 Hz, 1H), 3.46-3.40 (q, J= 20.1 Hz, 4H), 1.15-1.10 (t, J= 13.8 Hz, 6H). 13C NMR (CDCl3, 75 MHz): 163.70, 162.00, 161.32, 160.55, 156.00, 148.15, 140.58, 135.71, 134.61, 134.09,
132.53, 129.22, 127.86, 126.22, 123.94, 123.22, 115.31, 114.48, 108.71, 105.90, 79.24, 78.09, 75.17, 64.10, 56.35, 43.98, 12.29. TOF-HRMS (m/z) found (calcd.) for C33H27N3O10 (M): [M+H]^+, 626.1782 (626.1775).

Compound 8

Tris[(1-benzyl-1H,2,3-triazol-4-yl) methyl] amine (50 nmol), Copper (I) iodide (50 nmol), and Sodium Ascorbate (VcNa) (100 nmol) were mixed together in DMSO. Followed by Morpholinos (50 nmol) in water, small molecule (100 nmol) in DMSO. The mixture was shacked and incubated 15h. Separate on HPLC.

8c: MS (modify protein way) (m/z) found (calcd.): 5338.4 (5336.1).

Compound 9

Tris[(1-benzyl-1H,2,3-triazol-4-yl) methyl] amine (50 nmol), Copper (I) iodide (50 nmol), and Sodium Ascorbate (VcNa) (100 nmol) were mixed together in DMSO. Followed by Morpholinos (50 nmol) in water, small molecule (100 nmol) in DMSO. The mixture was shacked and incubated 15h. Separate on HPLC.

MS (modify protein way) (m/z) found (calcd.): 3995.6 (3996.1).

**Bifunctional molecule with fluorophore mimic**

![Bifunctional molecule with fluorophore mimic diagram]

Compound 3 was synthesized as described below,
5-Hexynoic acid (112 mg, 1 mmol), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, 230 mg, 1.2 mmol), and Et₃N (202 mg, 2 mmol) were stirred in DCM 30min. Then Compound 2 (374 mg, 1.2 mmol) was added to above solution. Stir overnight at room temperature. Purify on silica gel. 

Rᵣ = 0.4 (Ethyl acetate). Yield: 84 %. ¹H NMR (CDCl₃, 300 MHz): 8.55-8.53 (dd, J = 7.5, 0.9 Hz, 1H), 8.39-8.36 (d, J= 8.4 Hz, 1H), 8.22-8.19 (dd, J= 8.4, 0.9 Hz, 1H), 7.64-7.59 (d, J = 15.6 Hz, 1H), 7.12 (s, 1H), 6.50-6.47 (m, 2H), 4.17-4.11 (t, J= 15.0 Hz, 2H), 3.79-3.73 (m, 2H), 3.45-3.42 (m, 2H), 2.47-2.42 (t, J= 14.7 Hz, 2H), 2.26-2.21 (m, 2H), 1.94-1.88 (m, 2H), 1.72-1.69 (m, 2H), 1.47-1.39 (m, 2H), 0.98-0.93 (t, J= 14.7 Hz, 2H). ¹³C NMR (CDCl₃, 75 MHz): 175.68, 164.77, 164.32, 150.00, 134.41, 131.13, 129.75, 124.94, 122.83, 120.33, 109.95, 103.21, 83.10, 69.47, 46.55, 39.96, 38.97, 34.87, 30.35, 24.08, 20.45, 17.77, 13.89. TOF-HRMS (m/z) found (calcd.) for C₂₄H₂₇N₃O₃ (M): [M+H]⁺, 406.2129 (406.2131).

Compound 4

Tris[(1-benzyl-1H-1,2,3-triazol-4-yl) methyl]amine (50 nmol), Copper (I) iodide (50 nmol), and Sodium Ascorbate (VcNa) (100 nmol) were mixed together in DMSO. Followed by Morpholinos (50 nmol) in water, small molecule (100 nmol) in DMSO. The mixture was shacked and incubated 15h. Separate on HPLC.

4b: MS (modify protein way) (m/z) found (calcd.): 4529.9 (4529.1). 14MO- NXYA
Pull down assay

We purchased 3'-biotin-labeled 14-mer and 3'-biotin-labelled 25-mer MOs for pre-miR-21 from Gene-Tools. Total RNAs purified (using Oligotex mRNAs kit from QIAGEN, Hilden, Germany) from HeLa cells (known to express miR-21) at 37°C. 125 µl of streptavidin beads was taken and washed with washing buffer (10mM MgCl₂, 50mM Tris HCl pH 7.8, 150 mM KCl, 10% Glycerol, 0.1% NP40) twice. These beads were divided into three equal parts in three 0.5 ml tubes, mixed with 45.4 ul of incubation buffer (10mM MgCl₂, 50mM Tris HCl pH 7.8, 150 mM KCl, 10% Glycerol, 0.1% NP40 and 1U/µl RNase inhibitor) and incubated for 15 minutes at room temperature. To the first and second tubes, each of the 12 µl of 14MO-biotin (100 µM) and 25-MO-biotin (100 µM) were added respectively and to the third tube 12 µl of Biotin only (100 µM) was added. Each tube was incubated with 1/3rd of total RNAs at 37°C for two hours by shaking. Each sample was transferred to 1.5 ml tube, spinned down and removed supernatant liquid. The beads were washed three times with 1ml of washing buffer incubating for 5 minutes in each step at room temperature. Then each tube was washed with 1ml nuclease-free water before adding 500 µl of 3M Urea and incubate at room temperature with shaking for one hour. Each sample was centrifugated and taken out the supernatant (pull downed RNA) carefully and performed ethanol precipitation to recover total RNA pull downed by each sample.

Alpha-P-32 labelling of pre-miR-21/29b1

The sequence of hsa-pre-miR-21 was obtained from miRbase (http://www.mirbase.org/). The hsa-pre-miR-21 RNA was in-vitro transcribed from
template DNA with T7-promoter. DNA template for in-vitro transcription was made from following primers,

Briefly, forward primer with T7 promoter

5’-
GAAATTAATACGACTCCTAGTTGTCGGGATAGCTTATCAGACTGATGTTG
ACTGTGAATCTCATGGC-3’

And reverse primer

5’-TGTCAGACAGCCCATCGACTGTTGCTGTCCATGAGATTCAACAGTCAC-3’

Similarly, for hsa-pre-miR-29b1

Forward primer with T7 promoter

5’-
GAAATTAATACGACTCCTAGTTGTCGGGATAGCTTATCAGACTGATGTTG
TTAGATTTAAATAGTGATTGCTA-3’

And reverse primer

5’-
CCCCCAAGAACAATGTATTTCAAATGGTGCTAGACAATCCTATTTAAATCT-3’

0.8 μM each, were subjected to primer extension using Taq polymerase (5 U), dNTPs (0.2 mM), Taq polymerase buffer (10X) and MgCl2 (1 mM). The reaction mixture was denatured by heating at 95° C for 30 seconds followed by annealing at 55°-58° C for 1 minute and primer extension incubation at 68° C for 1 minutes. This cycle was repeated 30 times before going to final extension at 68° C for 5
minutes. The hybrid template with T7 promoter was checked using Agrose gel and used for in vitro transcription with alpha-P\textsuperscript{32}-UTP following manufacturer's instructions (Ambion Inc.). The pre-miR-21 substrate was purified by NucAway Spin Columns (Ambion Inc.) and checked on 16 % denaturing PAGE.

**Dicer cleavage inhibition assay**

\textsuperscript{32}P-labeled pre-miRNA was prepared as described earlier. A 10 µL of the reaction mixture was made by incubating \textsuperscript{32}P-labeled pre-miR-155 (1 µL, ~20 ng) with Dicer enzyme (Gen-Lantis) (1 µL) and various concentrations of compounds in buffer (HEPES 24 mM, NaCl 200 mM, EDTA 0.04 mM, MgCl\textsubscript{2} 2.5 mM, ATP 1 mM, pH 7.5) at 37 °C for 2.5 h. The reaction was stopped by boiling with equal volume of Gel Loading Buffer II (Thermo-Fisher Scientific) for 5 min. The non-cleaved pre-miRNA and the processed miRNA were resolved by 18% denaturing polyacrylamide gel. The gel was imaged with phosphor imager and analyzed by quantity one software (Bio-Rad).
2.5 References


14. Estimated Tm predicted and provided by GeneTools, LLC.


Chapter 3

Optimization of bi-functional regulators and cellular delivery

3.1 Introduction

After showing bi-functional strategy works, we next focused on optimizing each functional unit of our bi-functional molecules and the cellular delivery. We tested the effects of additional Dicer inhibition molecules and other ASOs as well as testing attaching the cell penetrating peptide (CPP) to facilitate the delivery.

3.2 Results and discussions

Optimization of the inhibition unit

Although the N-hydroxyl phthalimide (compound 1) worked as the Dicer inhibition unit in our bi-functional regulator, we expected that other more potent RNase III inhibitors (e.g. N-hydroxyimides, diketobutanoates or Flutimides, 4, 5 and 6) may enhance the activity of bi-functional regulators. To test this, we synthesized compound 20 containing a more potent inhibitor and a short linker and conjugated it to the 12-mer and 14-mer MOs designed for pre-miR-21 to give new bi-functional regulators (compound 21 and 22) (Scheme 3.1).

Scheme 3.1: Synthesis of bi-functional molecule with more potent inhibitor

21 = 12 MO-21
22 = 14 MO-21
Another strategy to enhance the activity of the Dicer inhibition unit is based on the crystal structure of Dicer with a double-stranded RNA, which shows two different cutting sites separated by 17.5 Å, involved in the cleavage of two RNA strands (Figure 3.1). We postulated that a Dicer inhibition unit with two inhibitors can inhibit both the active sites simultaneously giving more potent inhibition. We designed and synthesized new Dicer inhibition units containing two compounds with different linker lengths (compounds 23 and 24 in Scheme 3.2 and 3.3) and then tested their Dicer inhibition activity.

![Figure 3.1: Crystal structure of Giardia Dicer. (A) Front and side view of Dicer showing the N-terminal platform domain (blue), the PAZ domain (orange), the connector helix (red), the RNase IIIa domain (yellow), the RNase IIIb domain (green)¹](image)

¹ Image reference for Figure 3.1
Scheme 3.2: Synthesis of two head inhibitor bi-functional regulator with short linker lengths

Scheme 3.3: Synthesis of two head inhibitor bi-functional regulator with long linker lengths
Dicer inhibition assay of optimized inhibitor modules and bifunctional molecules

We compared the activity of new Dicer inhibition units we synthesized with the previously used compound 7 by the Dicer cleavage assay. All the new inhibitors showed enhanced activity as compared to compound 7. The new inhibitor unit with N-hydroxy imides (compound 20) shows at least four times better activity as compared to compound 7 and compound 24 with two units of compound 7 connected by a longer linker shows at least 10 times improved activity (Figure 3.2).

Figure 3.2: Dicer cleavage assay for optimized inhibition modules A) and B) Gel electrophoresis of cleaved and un-cleaved pre-miR-21 C) and D) quantification of pre-miR-21 band in each lane (3-independent experiments were used to calculate the error bar) for A and B.
We next tested the activity of bi-functional molecules, compounds 21 and 22, which contain N-hydroxy imide (compound 20) as the Dicer inhibition unit and compared to the original bi-functional molecule (compound 12) using the Dicer cleavage assay. Unexpectedly, we didn’t observe any enhanced activity from new bi-functional regulator with more potent inhibitor unit (Figure 3.3). Furthermore, from compound 22 and 21 with 14-mer and 12-mer MO respectively also showed similar activity. In addition, we synthesized bi-functional molecules incorporating compound 23 and 24 as the inhibition units and tested their activity, which

![Figure 3.3: Dicer cleavage assay for optimized bifunctional molecules A) and B) Gel electrophoresis of cleaved and un-cleaved pre-miR-21 C) and D) quantification of pre-miR-21 band in each lane (3-independent experiments were used to calculate the error bar) for A and B respectively](image-url)
unfortunately did not show any inhibition. Overall, our attempts to improve the activity of bi-functional molecules by enhancing the activity of Dicer inhibition unit have not been successful.

**Effect of the presence of other RNAs on regulator activity**

In the cellular environment, many other RNA species exist that may also bind the bi-functional regulators due to the imperfect pairing with the MOs used in the bi-functional molecule. This may lead to a reduced activity in cells as we observed than reflected in the Dicer cleavage assay performed previously, which only contain the pre-miR-21. To find out whether the presence of other RNAs as in cells diminishes the activity of our bifunctional molecules, Dicer cleavage assays were carried out in absence and presence of equivalent total RNA. We observed that the presence of total RNA has almost no effect on the potency of our bi-functional molecule on the processing of pre-miR-21 (**Figure 3.4**), which indicates that the lack of cellular activity observed previously is likely not due to this possibility.

![Figure 3.4: Dicer cleavage assay for bi-functional molecules in presence of total RNA](image)

A) Gel electrophoresis of cleaved and un-cleaved pre-miR-21  B) quantification of pre-miR-21 band in each lane (3-independent experiments were used to calculate the error bar) for A
Optimization of recognition module

Since the optimization on the Dicer inhibition units did not offer increased activity of the bi-functional molecules, we next focused on the change of the pre-miRNA recognition unit. Peptide Nucleic Acids (PNAs), are ASOs with phosphodiester backbone replaced by N-(2-aminoethyl) glycine units. PNAs have high binding affinity, are resistant towards enzymatic degradation and easier to synthesize. However, their neutral backbone cause decreased aqueous solubility.\textsuperscript{7-20}

![Chemical Structures of DNA (RNA), PNA, and MP-Containing γPNA Units](image)

Figure 3.5: Chemical Structures of DNA (RNA), PNA, and MP-Containing γPNA Units\textsuperscript{20}

Our collaborator, Prof. Danith Ly at Carnegie Mellon University has developed a new class of PNAs incorporating conformationally preorganized, diethylene glycol-containing γPNAs which shows enhanced binding affinity with DNA/RNA and water
solubility (Figure 3.5 and 3.6). To test if yPNAs would serve as better recognition units, we designed 7-nt (FL1), 9-nt (FL2) and 11-nt (FL3) yPNA oligonucleotides with 3'-azide modification to target the loop region of pre-miR-21. FL4 (11-mer) was designed as a negative control that cannot recognize pre-miR-21.

**y-PNA**

<table>
<thead>
<tr>
<th>FL1</th>
<th>ACAACTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>FL2</td>
<td>ACAACTTAG</td>
</tr>
<tr>
<td>FL3</td>
<td>ACAACTTAGAG</td>
</tr>
</tbody>
</table>

Figure 3.6: Synthesis of R-MPyPNA Monomers
To find out if these γPNAs bind pre-miR-21, gel shift assays were performed. We

Figure 3.7: Gel Shift assays for γPNA oligonucleotides

Figure 3.8: Dicer cleavage assay for γPNAs A) Gel electrophoresis of cleaved and un-cleaved pre-miR-21 and pre-miR-29b1 B) quantification of pre-miR-21 (lane 1-6) and pre-miR-29b1 (lane 7-12) band in each lane (3-independent experiments were used to calculate the error bar) for A
incubated P32 labelled pre-miR-21 with each γPNA oligonucleotide for 1 h and analyzed by 15% nondenaturing polyacrylamide gel followed by exposing to photographic screen and detected using BioRad PMI. Only FL2 (9-mer) and FL3 (11-mer) were found to be able to bind pre-miR-21, whereas the shorter γPNA oligonucleotide (FL1, 7-mer) and negative control show no binding affinity (Figure 3.7). The Dicer cleavage assays were then performed to determine the inhibitory activity of γPNAs. We observed that both FL2 and FL3 show certain activity especially the longer γPNA (FL3) and such inhibition is specific to miR-21 (Figure 3.8). We coupled FL3 N-hydroxy imide (compound 18) using click chemistry to give a bi-functional molecule FL3-6-R (compound 25). Dicer cleavage inhibition assay was then carried out with γPNA (FL3) alone or the bi-functional molecule (compound 25). We observed that the bi-functional molecule indeed has increased activity than individual units (Figure 3.9).

![Figure 3.9: Dicer cleavage assay for γPNA-bifunctional molecule A) Gel electrophoresis of cleaved and un-cleaved pre-miR-21 B) quantification of pre-miR-21 band in each lane (3-independent experiments were used to calculate the error bar) for A](image)
Comparative inhibition potency of bi-functional molecules

To compare if the bi-functional regulator with yPNA has increased activity than MO, we calculated the half inhibitory concentration (IC50) of compound 12 (with N-hydroxy phthalimide inhibitor and 14-me MO), compound 22 (with N-hydroxy imide and 14-mer MO) and compound 25 (with N-hydroxy imide and yPNA). Dosage dependent Dicer cleavage assays were carried out for each molecule from low to high concentrations under similar experimental condition and IC50 value was obtained for each bifunctional molecule.

Figure 3.10: IC50 value of three most potent bi-functional molecules (three independent Dicer cleavage inhibition assays were carried out with series dilution for each molecule to calculate errors)
IC50 values for compounds 12, 22 and 25 were found to be 4.2 µM, 3.5 µM and 0.5 µM (Figure 3.10). From these results, we found that optimizing the pre-miRNA recognition unit can potentially offer a way to improve the activity of the bi-functional regulators.

**Using CPPs to facilitate the cellular delivery of bi-functional molecules**

Since Endo-porter had failed to efficiently deliver the bi-functional regulators, we explored the possibility of incorporating CPPs (oligopeptides typically less than 30 aa residues and net positive charge) to enhance cellular delivery (Table 3.1).²³-²⁸ CPP-mediated transport system has been used successfully to deliver cargos (e.g. ASOs, siRNAs, antibodies, proteins) into the cells (Figure 3.11).²⁷

![Figure 3.11: CPP-conjugated molecule delivery across the cell membrane](image)

Figure 3.11: CPP-conjugated molecule delivery across the cell membrane²⁷
We chose to incorporate the commonly used polyarginine (R8) peptide (Figure 3.12) to deliver our bi-functional molecule. To test if R8 can be used to deliver MO, we synthesized a fluorescently labeled model bi-functional molecule incorporating a cysteine (Cys)-conjugated CPP in bifunctional molecule through a disulfide bond (compound 26, Scheme 3.4). Under the reducing environment in cells, it is expected that the disulfide bond will be cleaved to give free bifunctional regulator.\textsuperscript{29,30}

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Length</th>
<th>Charge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penetratin</td>
<td>RQIKIWFQNRMKWKK</td>
<td>16</td>
<td>7(+    )</td>
</tr>
<tr>
<td>TAT</td>
<td>GRKKRRQRRRPQ</td>
<td>13</td>
<td>8(+    )</td>
</tr>
<tr>
<td>Transportin</td>
<td>GWTLNSAGYLLGKINLKALAALAKKIL</td>
<td>27</td>
<td>5(+    )</td>
</tr>
<tr>
<td>R9 peptide</td>
<td>RRRRRRRRR</td>
<td>9</td>
<td>9(+    )</td>
</tr>
<tr>
<td>MPG peptide</td>
<td>GALFLGWLGAAGSTMGAPKKRKV</td>
<td>24</td>
<td>5(+    )</td>
</tr>
<tr>
<td>KALA peptide</td>
<td>WEAKLAKALAKALAKHLAKALAKKACEA</td>
<td>30</td>
<td>7(+    )</td>
</tr>
</tbody>
</table>

Table 3.1: Most commonly used CPPs\textsuperscript{23-28}

Figure 3.12: CPP (R8) used to deliver bi-functional mimic
We examined the cellular delivery of molecule 26 in Hela cells and found that R8 CPP significantly increased the delivery efficiency as compared to the delivery by Endo-porter (Figure 3.13).
Synthesis of CPP-conjugated bifunctional molecule was attempted by incorporating 3’-azide as well as protected sulfohydryl group at 5’-end in Morpholino recognition module, but several synthetic problems have been encountered, including the low purity of modified Morpholino from Gene-Tools.

Figure 3.13: Comparison between cellular delivery by CPP-conjugated system vs Endo-porter A) Delivery of molecule by CPP-conjugation at different concentration B) Delivery of molecule without conjugating CPP and using Endo-porter (all the images were taken after washing out media)
3.3 Conclusion

In this chapter, we investigated different strategies to optimize our bifunctional regulators. We found that incorporating more potent inhibition units did not increase the potency of bi-functional molecules significantly. One potential reason may be that since, bifunctional design specifically enriches the Dicer inhibitor unit at the active site of Dicer associated with targeted miRNAs, the local concentration of inhibitor might be already very high that even a less potent inhibitor can be sufficient to inhibit Dicer activity.

However, our attempts to employ a more potent ASO as the pre-miRNA recognition unit did lead to a more potent bi-functional molecule. We switched MOs to PNA as the recognition unit and were able to decrease their lengths from 14-mer in MO to 11-mer in PNA. The resulting PNA-based bi-functional molecule 25 has shown at least 7-fold increase in potency as compared to other MO-based bifunctional molecules (compounds 12 and 22).

Finally, we showed that R8 CPP can efficiently deliver a model MO-based bi-functional probe, which can be used for future delivery of the bifunctional regulators into the cells.
3.4 Methods

Optimized inhibition modules synthesis

![Chemical structure diagrams]

Compound 1

To a stirred solution of 2-(carboxymethyl)-5-hydroxybenzoic acid (1.00 g) in MeOH (50 mL) was added sulfuric acid (200 μL) dropwise. The mixture was refluxed for 4 h. The solvents were removed by vacuum and the residue was partitioned between water (50 mL) and ethyl acetate (50 mL). The organic phase was washed with water (2 X 50 mL) and dried over Na2SO4. The solid was removed by filtration. The filtrate was vacuumed to remove the solvent. The crude product was purified by chromatography (Hexane/Ethyl Acetate: 1/1) to give 1 as a yellow oil (0.98 g, 86%). 1 H NMR (300 MHz, DMSO): δ (ppm) 9.76 (s, 1H), 7.31 (d, J = 2.7 Hz, 1H), 7.16 (d, J = 8.4 Hz, 1H), 6.92 (dd, J = 8.4 Hz, 2.7 Hz, 1H), 3.84 (s, 2H), 3.75 (s, 3H), 3.57 (s, 3H).

Compound 2

To a stirred solution of 1 (200 mg, 0.89 mmol) in DMF (5 mL) was added K2CO3 (370 mg) and propargyl bromide (400 mg, 80% in toluene, 2.68 mmol). The reaction was stirred overnight at 70 °C. Water (50 mL) was added. The mixture was extracted with ethyl acetate (2 X 50 mL). The combined organic phase was washed with brine (3 X 50 mL) and dried over Na2SO4. The solid was filtered off.
The filtrate was vacuumed to remove the solvent. The residue was further purified by chromatography (Hexane/Ethyl Acetate: 5/1) to give 2 as a yellow oil (0.195 g, yield 83%). 1 H NMR (300 MHz, MeOD): δ (ppm) 7.59 (d, J = 2.7 Hz, 1H), 7.24 (d, J = 8.4 Hz, 1H), 7.15 (dd, J = 8.4 Hz, 2.7 Hz, 1H), 4.77 (d, J = 2.4 Hz, 2H), 3.94 (s, 2H), 3.84 (s, 3H), 3.66 (s, 3H), 2.97 (t, J = 2.4 Hz, 1H). 13 C NMR (300 MHz, MeOD): δ (ppm) 174.2, 168.6, 158.1, 134.6, 131.8, 130.1, 120.0, 118.1, 79.4, 77.2, 56.8, 52.5, 52.3, 40.3. HRMS (ESI) m/z [M + Na]+ 285.0731, calculated for C14H14O5Na 285.0739

Compound 3

To a solution of 2 (1 eq) in methanol (5 mL) was added water (5 mL) and LiOH·H2O (4 eq). The reaction was stirred overnight at room temperature. The solvents were removed by vacuum. The residue was dissolved in water (10 mL). Concentrated HCl (2 mL) was added to adjust the pH to 1, resulting in white precipitation. The solid was collected by filtration and dried to give the product as a white solid. 290 mg of 2 was used to produce 187 mg of 3 (white solid, yield 72%). 1 H NMR (300 MHz, MeOD): δ (ppm) 7.63 (d, J = 2.7 Hz, 1H), 7.23 (d, J = 8.4 Hz, 1H), 7.14 (dd, J = 8.4 Hz, 3.0 Hz, 1H), 4.77 (d, J = 2.4 Hz, 2H), 3.95 (s, 2H), 2.96 (t, J = 2.4 Hz, 1H). 13 C NMR (75 MHz, MeOD): δ (ppm) 175.8, 170.0, 157.9, 134.5, 132.4, 130.7, 119.8, 118.4, 79.5, 77.1, 56.8, 40.4. HRMS (ESI) m/z [M + H]+ 233.0451, calculated for C12H9O5 233.0450.

Compound 4
A solution of 3 (1 eq) and O-(4-Methoxybenzyl)-hydroxylamine (1.2 eq) in toluene (180 mL) was refluxed using a Dean-Stark apparatus overnight. The solvent was removed by vacuum and the residue was purified by column chromatography. Compound 3 (187 mg) was used to give 4 (174 mg) as a white solid. Elution solvent: Hexane/Ethyl acetate 2/1; yield 62%. 1 H NMR (300 MHz, CDCl3): δ (ppm) 7.76 (d, J = 2.1 Hz, 1H), 7.52 (d, J = 8.7 Hz, 2H), 7.22 (m, 2H), 6.90 (d, J = 8.7 Hz, 2H), 5.08 (s, 2H), 4.77 (d, J = 2.1 Hz, 2H), 4.07 (s, 2H), 3.81 (s, 3H), 2.56 (t, J = 2.4 Hz, 1H). 13 C NMR (75 MHz, CDCl3): δ (ppm) 166.0, 161.5, 160.4, 157.2, 131.8, 128.9, 126.4, 126.2, 122.9, 114.0, 112.8, 78.1, 77.8, 76.4, 56.3, 55.4, 37.0. HRMS (ESI) m/z [M + Na]+ 374.1008, calculated for C20H17NNaO5 374.1004.

Compound 5

To a stirred solution of 4 (27 mg, 0.077 mmol) in DCM (1.5 mL) was added TFA (1.5 mL). The reaction turned to dark red after 4 h. The solvents were removed by vacuum. The residue was washed with ether (3 X 1.5 mL) to give 5 as a white solid (15 mg, yield 81%). 1 H NMR (300 MHz, DMSO): δ (ppm) 10.39 (s, 1H), 7.57 (d, J = 2.4 Hz, 1H), 7.31 (m, 2H), 4.90 (d, J = 2.1 Hz, 2H), 4.18 (s, 2H), 3.60 (t, J = 2.1 Hz, 1H). 13 C NMR (75 MHz, DMSO): δ (ppm) 166.4, 161.5, 156.2, 129.1, 127.5, 126.0, 121.7, 111.9, 78.8, 78.7, 55.7, 36.2. HRMS (ESI) m/z [M + H]+ 232.0616, calculated for C12H10NO4 232.0610

Compound 6
Tris[(1-benzyl-1H-1,2,3-triazol-4-yl) methyl] amine (50 nmol), Copper (I) iodide (50 nmol), and Sodium Ascorbate (VcNa) (100 nmol) were mixed together in DMSO. Followed by Morpholinos (50 nmol) in water, small molecule 5 (100 nmol) in DMSO. The mixture was shaken and incubated 15h. Separate on HPLC.

6a (with 12 MO-21) = MS (modify protein way) (m/z) found (calcd.): 4516.34 (4516.21).

6b (with 14 MO-21) = MS (modify protein way) (m/z) found (calcd.): 5170.64 (5171.21).

**Synthesis of FL3 bi-functional molecule**

Tris[(1-benzyl-1H-1,2,3-triazol-4-yl) methyl] amine (50 nmol), Copper (I) iodide (50 nmol), and Sodium Ascorbate (VcNa) (100 nmol) were mixed together in DMSO, followed by FL3 (50 nmol) in water, small molecule 5 (100 nmol) in DMSO. The mixture was shaken and incubated 5h, Separated on HPLC to get FL3-6R bifunctional molecule. MS = m/z found (calcd.) 4695.01 (4696.57)
Compound 2

To a stirred solution of dimethyl 5-hydroxyisophthalate (DMHIP, 6.3 g, 30 mmol) and propargyl bromide (80% in toluene, 5.0 g, 33 mmol) in DMF (100 mL) were added potassium carbonate (4.2 g, 30 mmol). The reaction mixture was stirred at 90°C about 2h. After the reaction finished, extract with Ethyl acetate/brine. Combine organic solvent, dry with Na2SO4. Remove the solvent, White solid was obtained. 1H NMR (CDCl3, 300 MHz): 8.33-8.32 (t, J= 3.0 Hz, 1H), 7.83-7.82 (d, J = 1.5 Hz, 2H), 4.79-4.78 (d, J= 2.4 Hz, 2H), 3.94 (s, 6H), 2.56-2.54 (t, J= 4.5 Hz, 1H).

Compound 3

LiAlH4 (1.6 g, 42 mmol) and THF (40 mL) was added in a 250-ml flask, the mixture was kept in ice bath and stirred for 10 min. DMPIP (4.5 g, 20 mmol) in THF (60 mL) was dropped into the flask. The reaction stood in ice bath for 1 h, then at room temperature for 7 h. After that, the reaction was quenched with water, and the mixture was filtrated and dried with anhydrous MgSO4. After the removal of solvent by rotary evaporation, the crude product was crystallized in hexane to give 1,3-dihydromethyl-5-proparyloxybenzene (DHMPB) as white crystals. 1H NMR (Acetone-d6, 500 MHz): 6.96 (s, 1H), 6.89 (s, 2H), 4.76 (d, J = 2.0 Hz, 2H), 4.60-4.59 (d, J= 5.5 Hz, 4H), 4.32-4.30 (t, J= 10.1 Hz, 2H), 3.05 (s, 1H). TOF-HRMS (m/z) found (calcd.) for C11H12O3 (M): [M+Na]⁺, 215.0674 (215.0684).

Compound 4 was made following the routine methods.

Compound 5
1H NMR (DMSO-d6, 500 MHz): 11.02 (s, 2H), 8.40-8.38 (d, J= 8.4 Hz, 2H), 8.21 (s, 2H), 7.97-7.96 (d, J = 7.5 Hz, 2H), 7.25 (s, 1H), 7.15 (s, 2H), 5.40 (s, 4H), 4.85 (s, 2H), 3.56 (s, 1H). 13C NMR (CDCl3, 75 MHz): 175.68, 164.77, 164.32, 150.00, 134.41, 131.13, 129.75. TOF-HRMS (m/z) found (calcd.) for C29H18N2O11 (M): [M+H]+, 571.1009 (571.0989); [M+Na]+, 593.0830 (593.0808).

Compound 6

Tris[(1-benzyl-1H-1,2,3-triazol-4-yl) methyl] amine (50 nmol), Copper (I) iodide (50 nmol), and VcNa (100 nmol) were mixed together in DMSO. Followed by Morpholinos (50 nmol) in water, small molecule (100 nmol) in DMSO. The mixture was shacked and incubated 15h. Separate on HPLC.

MS (modify protein way) (m/z) found (calcd.): 5510.8 (5510.1). 14MO-DI-PA

Compound 2

Into a solution of 1 in THF (10 ml), SOCl2 and DMF were added. The reaction was refluxed for 2 h. The solvent was removed by vacuum and residue was dissolved in THF (5 ml) and the THF was removed by vacuum to get compound 2.
Compound 4

Into a solution of 3 in DCM (5 ml), TEA was added. The reaction was cooled to 0°C. A solution of 2 in DCM (5 ml) was then added dropwise. The reaction was allowed for warming to room temperature and stirred for overnight. The reaction was washed with H₂O (1x10 ml) and dried over Na₂SO₄. The solvent was removed by vacuum. The residue was purified by chromatography (Hexane: EA=3:1) to give the product 4 as a white solid (81%).

Compound 5

Into a solution of 4 in DCM, TFA was added. The reaction was stirred for 3 h. The solvent was removed by vacuum. CH₃CN (5 ml) was added to the residue and then removed by vacuum. It was repeated for 4 times and the residue was then titrated with Et₂O to form white solid. It was collected by filtration and dried over vacuum to give the product as a white solid (100%). ¹H NMR (300 MHz, D₂O, δ) 8.22 (m, 1H), 8.12 (d, J = 7.5 Hz, 1H), 7.11 (m, 1H), 7.30 (m, 5H), 5.03 (s, 2H), 4.33 (br, 2H), 3.07 (t, J = 7.2 Hz, 2H), 2.07 (m, 2H). HRMS (ESI) m/z [M+H]⁺ = 355.1290 (M+1)⁺

Compound 7

Same as in compound 2

Compound 8

Into a solution of 5 and 7 in DCM, TFA was added at 0°C. The reaction was stirred for 12 h. Then the reaction was diluted with DCM (10 ml) and washed with water
(3X 10 ml). the organic phase was purified by chromatography (MeOH: DCM=1:30) to give the product 8 (76%).$^1$H NMR (300 MHz, CDCl$_3$, δ) 8.32 (m, 4H), 7.77 (m, 3H), 7.50 (m, 6H), 7.36 (m, 6H), 7.10 (t, $J = 5.7$ Hz, 2H), 5.21 (s, 4H), 4.69 (d, $J = 2.1$ Hz, 2H), 4.46 (t, $J = 5.7$ Hz, 4H), 3.58 (q, $J = 6.0$ Hz, 4H), 2.57 (t, $J = 2.1$ Hz, 1H), 2.10 (t, $J = 6.0$ Hz, 4H). HRMS (ESI) m/z [M+H]$^+$ = 893.2669 (M+1)$^+$

Compound 9

Compound 8 was dissolved in CH$_3$SO$_3$H and stirred for 2 h at room temperature. 5ml of water was added to the reaction, and white solid was obtained and collected by centrifugation and purified by prep-TLC (MeOH: DCM=1:2) to give the product as a white solid (46%).$^1$H NMR (300 MHz, DMSO, δ) 11.03 (br, 2H), 8.67 (t, $J = 4.8$ Hz, 2H), 8.34 (m, 2H), 8.19 (t, $J = 0.6$ Hz, 2H), 7.92 (m, 3H), 7.53 (d, $J = 1.2$ Hz, 2H), 4.86 (d, $J = 2.4$ Hz, 2H), 4.39 (t, $J = 6.0$ Hz, 4H), 3.60 (s, 1H), 3.58 (m, 4H), 2.00 (q, $J = 6.0$ Hz, 4H). HRMS (ESI) m/z [M+H]$^+$ = 713.1750 (M+1)$^+$

**Alpha-P32-UTP labelling of pre-miR-21/29b1**

The sequence of hsa-pre-miR-21 was obtained from miRbase ([http://www.mirbase.org/](http://www.mirbase.org/)). The hsa-pre-miR-21 RNA was *in-vitro* transcribed from template DNA with T7-promoter. DNA template for in-vitro transcription was made from following primers,

Briefly, forward primer with T7 promoter
5' -
GAAATTAATACGACTCACTATAGGTGTCGGGTAGCTTATCAGACTGATGTTGACTTTGAATCTCATGGC-3'

And reverse primer

5' -
TGTCAGACAGCCCATCGACTGGTGTTGCCATGAGATTCAACAGTCAAC-3'

Similarly, for hsa-pre-miR-29b1

Forward primer with T7 promoter

5' -
GAAATTAATACGACTCACTATAGGTGTCGGGTAGCTTATCAGACTGATGTTGTTAGATTTAAATAGTGATTGTCTA-3'

And reverse primer

5' -
CCCCCAAGAACACTGATTTCAAATGGTGCTAGACAATCACTATTTAAATCTTATCT-3'

0.8 μM each, were subjected to primer extension using Taq polymerase (5 U), dNTPs (0.2 mM), Taq polymerase buffer (10X) and MgCl2 (1 mM). The reaction mixture was denatured by heating at 95° C for 30 seconds followed by annealing at 55°-58° C for 1 minute and primer extension incubation at 68° C for 1 minutes. This cycle was repeated 30 times before going to final extension at 68° C for 5 minutes. The hybrid template with T7 promoter was checked using Agrose gel and used for in vitro transcription with alpha-P32-UTP following manufacturer's
instructions (Ambion Inc.). The pre-miR-21 substrate was purified by Nuc-Away Spin Columns (Ambion Inc.) and checked on 16 % denaturing PAGE.

**Dicer cleavage inhibition assay**

$^{32}$P-labeled pre-miRNA was prepared as described earlier. A 10 µL of the reaction mixture was made by incubating $^{32}$P-labeled pre-miR-155 (1 µL, ~20 ng) with Dicer enzyme (Gen-Lantis) (1 µL) and various concentrations of compounds in buffer (HEPES 24 mM, NaCl 200 mM, EDTA 0.04 mM, MgCl$_2$ 2.5 mM, ATP 1 mM, pH 7.5) at 37 °C for 2.5 h. The reaction was stopped by boiling with equal volume of Gel Loading Buffer II (Thermo-Fisher Scientific) for 5 min. The non-cleaved pre-miRNA and the processed miRNA were resolved by 18% denaturing polyacrylamide gel. The gel was imaged with phosphor imager and analyzed by quantity one software (Bio-Rad).

**CPP-conjugated bifunctional mimic with fluorophore synthesis**

N-Propargyl-4-amido-1,8-naphthalimide (3) was synthesized according literature reported procedures*.

*Chemical Communications (Cambridge, United Kingdom), 52(89), 13086-13089; 2016

**Compound 1**

2,2'-Dipyridyldisulfide (1.21g, 6.0mmol) was dissolved in MeOH (3ml) and AcOH (0.13ml) was added. To this mixture, a solution of 2-mercaptoethanol (0.3ml, 4.4mmol) in MeOH (3ml) was added dropwise at room temperature over 30 mins
period under continuous stirring. The reaction mixture was further stirred at room
temperature overnight. The solvent was evaporated and residue was purified by
silica gel column chromatography (EtOAc: Hexane, 1:5 to 1:1), separating the
product as a colorless oily liquid (0.56g, 68.1% yield). ¹H NMR (300 MHz, CDCl₃)
δ ppm 8.502-8.485(m, 1H), 7.616-7.545(m, 1H), 7.409-7.375(m, 1H), 7.164-
7.119(m, 1H), 5.753(s, 1H), 3.811(t, 2H, J=4.8Hz), 2.959-2.925(m, 2H).
Compound 4:

To a stirred solution of 3 (0.195g, 0.78mmol) and DIPEA (0.300g, 2.3mmol) in dry dichloromethane (5ml), a phosgene solution (15 wt. % in toluene, 3.64ml, 5.1mmol) was added. The reaction mixture was stirred at 0°C under inert condition. After 2h, the excess phosgene was removed from the solution by Argon. Then compound 1 (0.3g, 1.6mmol) in dry dichloromethane (2ml) and DMAP (0.244g, 2.0mmol) was added to the reaction mixture and was stirred over-night. After completion of reaction (by TLC), the mixture was diluted with EtOAc (10ml), washed twice with brine solution, dried over sodium sulfate. Filtered and the residue was purified by column chromatography (EtOAc: CH2Cl2 1:10). Light green solid was purified (0.36g, 63.1% yield). 1H NMR (300 MHz, CDCl3) δ ppm 8.705(dd, 2H, J=7.2, 12.9Hz), 8.470-8.454(m, 1H), 8.389(d, 1H, J= 8.4Hz), 8.287(d, 1H,J= 8.7Hz), 7.837-7.784(m, 1H), 7.720-7.583(m, 3H), 7.114-7.074(m, 1H), 4.965(d, 2H, J=2.4Hz), 4.582(t, 2H, J=6.0Hz), 3.185(t, 2H, J=6.0Hz), 2.200(t, 1H, J=2.4Hz).

Compound 5

The compound 4 (10ul, 10mM in DMSO) and Morpholino (50ul, 1mM in H2O) were mixed together. While stirring the solution, the CuSO4·5H2O/Sodium Ascorbate (VcNa) solution (10ul, 10mM aqueous solution) was added. After stirring the
mixture at rt for 12 h, the reaction is finished and purified by HPLC to purify the product as light-yellow solid.

\[ \text{MS} = \text{(modified protein way)} \; m/z \; [M+H]^+ = 4750 \; (M+1)^+ \]

**Compound 6**

The compound 5 (50ul, 0.153mM) was mixed with R8 peptide (8ul, 1mM) in tris buffer pH 8.0 (50ul, 1M). The solution was stirred at room temperature over-night. The mixture was purified by HPLC to give 6 as light yellow solid.

Fluorescence images of HeLa cells incubated with Compound 6 solution with excitation at 359nm,

**MO-FL-R8**
3.5 References


8. Torres, A. G., Threlfall, R. N., & Gait, M. J. Potent and sustained cellular inhibition of miR-122 by lysine-derivatized peptide nucleic acids (PNA) and phosphorothioate locked nucleic acid (LNA)/2′-O-methyl (OMe) mixmer anti-miRs in the absence of transfection agents. Artificial DNA, PNA & XNA, 2(3), 71–78 (2011)


Chapter 4

Explore alternative pre-miRNA binder

4.1 Introduction

Although ASOs like MOs or PNAs can be used for our bi-functional molecules, they have several limitations. To find alternative pre-miRNA binders to be used as

Figure 4.1: Biogenesis of miR-155 and downstream cellular function in breast cancer

1
our recognition unit, we focused on the cyclic peptides, which have been shown to recognize RNAs with great cell permeability and stability. In this chapter, we tested the activity and selectivity of a novel cyclic peptide scaffold to target miR-155, which is a known oncogenic miRNA found to also play roles in cardiovascular diseases, immunity disorders, inflammation, and viral infections (Figure 4.1).\textsuperscript{2-16} MiR-155 is upregulated in different breast cancer tissues and used as a pathological marker, representing tumor condition and survival rate (Table 4.1).\textsuperscript{1} It has been shown that over expression of miR-155 directly promote tumor formation and pharmacological intervention causes tumor regression.\textsuperscript{1} As a result, miR-155 becomes an important therapeutic target.

<table>
<thead>
<tr>
<th>miR-155</th>
<th>Tissue type</th>
</tr>
</thead>
<tbody>
<tr>
<td>↑ in breast cancer</td>
<td>76 Breast tumor</td>
</tr>
<tr>
<td>↑ in breast cancer</td>
<td>10 Normal breast</td>
</tr>
<tr>
<td>↑ in ER\textsuperscript{−} tumors</td>
<td>363 Breast tumor</td>
</tr>
<tr>
<td>↑ in malignant breast tissue</td>
<td>177 Normal breast</td>
</tr>
<tr>
<td>↑ in PR\textsuperscript{+} tumors</td>
<td>93 Breast tumor</td>
</tr>
<tr>
<td>↑ in grade II and III tumors</td>
<td>5 Normal breast</td>
</tr>
<tr>
<td>↑ in ER\textsuperscript{−} PR\textsuperscript{−} tumors</td>
<td>34 Breast tumor</td>
</tr>
<tr>
<td>Associated with higher tumor grade, advanced tumor stage, lymph node metastasis</td>
<td>6 Normal breast</td>
</tr>
<tr>
<td>↑ in 41 of 45 invasive</td>
<td>Serum-13 breast cancer patients, 8 healthy patients</td>
</tr>
<tr>
<td>↑ 2 of 17 noninvasive tumors</td>
<td>Tumor, normal adjacent tissue, and serum from 68 breast cancer patients</td>
</tr>
<tr>
<td>↑ in 55 breast tumors</td>
<td>Tissue and serum from 40 healthy patients</td>
</tr>
<tr>
<td>↑ in 31 recurrent tumors</td>
<td>92 Breast tumor and normal adjacent tissue</td>
</tr>
<tr>
<td>↑ in breast metastases</td>
<td>45 Invasive breast tumor</td>
</tr>
<tr>
<td>↑ in tumors</td>
<td>17 Noninvasive breast tumor</td>
</tr>
</tbody>
</table>

Table 4.1: miR-155 expression reported in different breast cancer cell types\textsuperscript{1}
4.2 Results and discussions

Our collaborator, Prof. Jianfeng Cai at University of South Florida, has recently developed one-bead–one-compound thioether bridged macrocyclic γ-AAPeptide combinatorial library containing novel class of the macrocyclic peptidomimetics (Figure 4.2). The γ-AAPeptides have modified peptide backbone with diverse functional side chains to introduce improved protease resistance, chemo-diversity, and bioavailability\textsuperscript{17,18}. To explore the possibility of using these peptidomimetics as a new class of RNA targeting molecules, we screened the macrocyclic γ-AAPeptide library against fluorophore-labelled pre-miR-155.

Figure 4.2: Synthesis of the thioether bridged one-bead–one-compound macrocyclic γ-AAPeptide library\textsuperscript{17,18}
Fluorophore labelling of pre-miRNA

Pre-miR-155 was *in-vitro* transcribed by using cDNA encoding premiR-155 sequence with T7 promoter and an extra G-nucleotide at 5’-end. During *in vitro* transcription GMPS (Guanosine mono phosphorothioate) was used along with normal ATP, UTP, CTP and GTP in 8:1 ratio\(^9\). The *in vitro* transcribed RNA will

![Diagram of transcription process](image1)

**Figure 4.3:** *In vitro* transcription followed by fluorophore labelling of pre-miRNA \(^{19,20}\) and gel image
have free thiol group at its 5’-end through the incorporation of GMPS. We then couple the ATTO 488 fluorophore to this free thiol group by reacting with the iodoacetamide.\textsuperscript{20}

To confirm the labeling, fluorophore labelled premiRNA-155 was analyzed by 16% polyacrylamide gel electrophoresis to visualize the fluorescence signal. Then same gel was also stained with Ethidium Bromide to image RNAs (Figure 4.3). The results showed that the pre-miRNA-155 was successfully labelled.

**Identification of pre-miR-155 binder and test its activity**

ATTO-488 labelled pre-miR-155 was screened against a library of macrocyclic peptidomimetics by Prof. Cai’s group. From the screening, compound 27 was discovered as a hit for pre-miR-155. After the structure was decoded, compound 27 was resynthesized and subjected to additional assays.

![Structure of library hit screened against pre-miR-155](image)

**Figure 4.4**: Structure of library hit screened against pre-miR-155

After we validated the binding using the fluorescence polarization assay, the \textit{in vitro} Dicer cleavage assay was carried out to evaluate the activity of compound
We observed that compound 27 can inhibit the cleavage of pre-miR-155 by Dicer at µM concentrations.

![Dicer cleavage assay](image)

**Figure 4.5:** Dicer cleavage assay for macrocyclic peptidomimetics (compound 27) A) Gel electrophoresis of cleaved and un-cleaved pre-miR-155 B) quantification of pre-miR-155 band in each lane (3-independent experiments were used to calculate the error bar) for A

**Evaluating targeting selectivity by RNAseq**

After we confirmed that compound 27 is active in blocking endogenous miRNA-155 production (carried out by another lab member), we investigated the targeting specificity of this new pre-miR-155 binder by RNAseq and bioinformatics analysis. We treated MCF-7 cells with compound 27, a commercial antimiR-155 (from IDT) as a positive control. Non-treated cells were used as the negative control and the standard for comparison. We performed three replicated experiments for each of these three conditions (Table 4.2) and total RNA from each experiment was isolated and sent for RNAseq performed by National Center for Genome Resources (NCGR).
<table>
<thead>
<tr>
<th>Sample</th>
<th>Condition</th>
<th>Replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Mcf7 cells only (non-treated)</td>
<td>A1, A2 and A3</td>
</tr>
<tr>
<td>B</td>
<td>Mcf7 cells treated with positive control antimiR-155-5p (IDT)</td>
<td>B1, B2 and B3</td>
</tr>
<tr>
<td>C</td>
<td>Mcf7 cells treated with our molecule (Compound 27)</td>
<td>C1, C2 and C3</td>
</tr>
</tbody>
</table>

Table 4.2: Description of experimental conditions for RNAseq experiment

The general process for RNAseq and analysis is illustrated in Figure 4.6 and 4.7. We were interested in finding out differential gene expression caused by downregulation of miR-155 and by any off-target effect from each treatment. After using Illumina sequencing platform to sequence all samples with each lane of 1X75nts, adapters were removed from raw read and trimmed depending on FastQC result. The coverage of sequencing was found to be very good for all samples with at least 24 million reads per sample and ranging up to 40 million reads per sample (Figure 4.8).
Figure 4.6: RNA isolation and library preparation for RNAseq\textsuperscript{21}
Figure 4.7: Bio-informatics analysis of sequenced reads\textsuperscript{21}
Trimmed reads from each sample were aligned with reference human genome using a tool called GSNAP (Genomic Short-read Nucleotide Alignment Program)\textsuperscript{22}.

Figure 4.8: The sequencing coverage for replicates of each sample

Figure 4.9: Alignment metrics for aligned samples
SAM files (Sequence Alignment Mapping) obtained from each alignment from each sample was used to get alignment metrics, which showed a good alignment of reads with our reference genome and similar alignment pattern among replicates for all samples, ranging from 50% to 90% (Figure 4.9). Additionally, majority of aligned reads in our samples were uniquely aligned that means, these reads were aligned to only one position in reference giving a high confidence on expression analysis. After making sure our reads are aligned with reference genome well, each SAM file and reference human genome annotated file (gff file) is used to run HTseq\textsuperscript{23} to find out the read count and text file obtained for all the replicates were combined into a file and followed DESEQ2 work flow to measure differential gene expression\textsuperscript{24}.

PCA (principal component analysis) plot gives the characteristics of samples depending on nature of gene expression level. When we plotted PCA, we got a very clear separation of samples into three groups with replicates of each samples

![PCA Plot for all samples](image)

Figure 4.10: PCA Plot for all samples
clustered together. This signifies that the gene expression pattern in each replicate for our samples are comparable or alternatively, there is variation between the samples but not between the replicates (Figure 4.10).

We used MA plot to compare differential expression of genes in sample B (treated with antimiR-155, IDT) and C (treated with compound 27) as compared to control sample A (non-treated Cells only) as reference. In this plot fold change is calculated from read count value of genes as compared to reference sample gene count, and then it is transformed into log2 value, where any genes have fold change value equals to ‘1’, will have ‘0’ log2 value and these genes are not considered as differentially expressed genes. When log2 transformed values of fold change for all the genes are plotted against mean of normalized counts, all the genes with no differential expression will appear at the center (black dots in Figure 4.11: MA plot to compare the differential expression of genes at two different treatment conditions with cells only as reference (padj ≤0.1).
whereas differentially expressed genes will appear around negative or positive axis depending on whether these are upregulated or down regulated (red dots in Figure 4.11). First MA plot in Figure 4.11 (refA Vs B) gives the pattern of differentially expressed genes of antimiR-155 treated sample as compared to non-treated cell reference whereas second plot (refA vs C) gives the pattern of differentially expressed genes of compound 27 treated cells as compared to non-treated cell reference. If we look closely this result, it shows a very distinct characteristic as the antimiR-155 caused more fold change of differentially expressed genes that are upregulated whereas compound 27 caused more fold change of differentially expressed genes that are downregulated (Figure 4.11).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total number of differentially expressed genes</th>
<th>Positive log2FC (Up-regulated)</th>
<th>Negative Log2FC (Down regulated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RefA vs. B</td>
<td>1404</td>
<td>753</td>
<td>651</td>
</tr>
<tr>
<td>RefA vs C</td>
<td>1935</td>
<td>1025</td>
<td>910</td>
</tr>
</tbody>
</table>

Table 4.3: Number of Up- and Down-regulated genes in each condition (padj ≤0.05)

It was found that compound 27 caused more genes to change expression as compared to antimiR-155 (Table 4.3). When we compared the list of differentially expressed genes in both conditions to each other, 12.8% of the genes are common. Among upregulated genes 9.5% of the genes are found to be common whereas in downregulated genes 8.5% of the genes are found to be common (Figure 4.12)
miR-155 target prediction and validated database

To investigate if the genes changing expression upon treatment are miR-155 direct or downstream targets, we first compile the database of genes that are known or predicted to be miR-155 targets. We can then compare the RNAseq results with the database to validate the targeting as well as to identify new miR-155 targets and investigate the off targets of compound 27 and antimiR-155.

Two in silico prediction software were used to predict miR-155 targets. “TargetScanHuman7.2” is a prediction software used to predict miRNAs targets by...
the alignment of conserved 6, 7 or 8 nucleotides from the seed region of miRNA of interest with human 3' UTRs and their orthologs, as defined by UCSC whole-genome alignments as well as detected within open reading frames (ORFs)\textsuperscript{25,26}. Similarly, "miRDB" is an online database for miRNA target prediction and functional annotation. All the targets were predicted using bioinformatics tool called "MirTarget" and functionality of miRNAs were explored by computational method and literature mining\textsuperscript{27,28}. Target genes for miR-155 (both -5p and -3p strands) were predicted using TargetScanHuman7.2 and 2,873 genes were identified at 3'UTR region of human genome. Similarly, miRDB was used to predict miR-155 target genes and 551 genes were identified at 3' UTR region of human genome. We further analyzed by comparing these target genes obtained from two different

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{miR-155_TargetScan.png}
\caption{Comparison of miR-155 targets obtained from two different prediction tools (miR-155_Targetscan represents the targets obtained by using "TargetScanHuman7.2" tool and miR-155_miRDB represents the targets obtained by using "miRDB")}
\end{figure}
target prediction tools. Out of all predicted genes, 339 genes are common to both (Figure 4.13)

Furthermore, one more database (miRTarBase7.0) that has information about updated experimentally validated miRNA target genes was used to find out the list of genes that are experimentally validated as miRNA targets.\(^29\) It includes the genes that are validated as miRNA target by one or more experimental methods like qPCR, western blot, reporter assay and/or microarray etc. By using this database, we prepared the list of genes that are validated experimentally as miR-155 targets and 256 genes were found as validated miR-155 genes. When compared with common predicted genes from earlier two prediction tools, 45 genes were found to be same in both list of genes (Figure 4.14).

![Venn diagram](image)

**Figure 4.14:** Comparison between common miRNA targets predicted and experimentally validated (miR-155\_predicted represents the total common genes obtained by using two different prediction tools in Figure 4.13 and miR-155\_validated represents experimentally validated targets obtained from miRTarBase7.0)
We will use union of all genes including common (predicted + validated) to compare with our experimentally upregulated genes.

After generating the database, we selected differentially expressed genes with positive log2FC values (up-regulated) from each treatment condition and compare those with miR-155 target genes (predicted + validated) because all direct target genes of miR-155 should be upregulated with the repression of miR-155.

A good fraction of genes in both treatment conditions were found to be similar to predicted miR-155 target genes, but majority of genes in each case were not in the

![Venn Diagram](image)

**Figure 4.15**: Comparison of upregulated genes for both samples with target genes A) comparison of anti-miR induced upregulated genes with miR-155 target genes B) comparison of compound 27 induced upregulated genes with miR-155 target genes C) common upregulated genes between anti-miR induced and compound 27 induced upregulated genes D) comparison between common upregulated genes with miR-155 target genes
list of predicted genes. This suggest either these genes are still valid targets but not been predicted or reported or they might be the secondary and downstream targets of miR-155 target genes. It is also possible that these gene changes are due to off-target effects that are irrelevant to miR-155 (Figure 4.15).

To understand if there is any functional relationship among the changed genes and investigate what are the potential off targets, we carried out gene enrichment and pathway analysis of all the upregulated genes using Cytoscape-ClueGO software to cluster the functionally similar genes (Figure 4.16 and 4.17).
Analyzing the genes that are upregulated upon anti-miR-155 treatment, we found the genes can be clustered to several functional groups including defense response, cell surface receptor signaling pathway, defense response to virus, response to organic substance, regulation of sequence specific DNA binding transcription factor, regulation of NF-kappaB (Figure 4.16).
Analyzing the genes that are upregulated upon compound 27 treatment, we found the genes can be clustered to several functional groups including sterol biosynthesis, cytoplasmic vesicle, cytoplasmic part, endomembrane system organization, single-organism biosynthesis process (Figure 4.17).

By comparing functional cluster of upregulated genes, we found low similarity between these two treatment conditions (Figure 4.16 and 4.17) and each of these also have low overlaps with the predicted and validated miR-155 targets (Figure 4.15). All of these results suggest that both antimir-155 and compound 27 have significant and distinct off targets and effects. We are still in the process of further analyzing the data using additional bioinformatics tools.
4.3 Conclusion

We discovered compound 27, to be a new pre-miR-155 binder through a peptidomimetics library screening, which also show in-vitro and cellular activity. RNAseq analysis from samples of cells treated with compound 27 and antimir-155 showed that although a certain number of differentially expressed genes in both treatment conditions matched predicted miR-155 target genes, there are a large fraction of differentially expressed genes in both conditions showed no direct connection to miR-155 targets. Further analysis of these genes should give information regarding the off-target effects of both molecules.
4.4 Methods

**Alpha-P32-UTP labelling of pre-miR-155**

The sequence of pre-miR-155 was obtained from miRBase (http://www.mirbase.org/). The DNA template used for making pre-miR-155 RNA was generated by PCR primer extension. Briefly, forward primer:

5’-
GAAATTAATACGACTCATAAATGTGTAATGCTAATCGTGATAGGGGGTTT
TTGCCTCCAACTG-3’

and reverse primer:

5’-CTGTTAATGCTAATATGTAGGAGTCAAGTGGAGGCAAACCCCCTA-3’

0.8 μM of each were subjected to primer extension using Taq polymerase (Ambion) per the manufacture’s protocol. The extended dsDNA was purified with NucleoSpin gel and PCR clean-up kit (Macherey-Nagel). The hybrid DNA template with T7 promoter was used for in vitro transcription using T7 polymerase following manufacturer’s instructions (New England Biolabs). The reaction mixture was treated with DNase to digest the template DNA and then extracted with phenol/chloroform/isoamyl alcohol (25:24:1) (pH 6.7). The RNA was finally precipitated by ethanol and resuspended in water for storage at -20 °C. Right before use, the RNA was allowed to refold as follows: RNA was heated to 94 °C for 2 min and then cooled to 4 °C at a rate of 1 °C/s.
Preparation of fluorophore labeled pre-miR-155

5'-GMPS primed pre-miR-155 were first prepared by in vitro transcription as described above except that 5'-GMPS(Biolog):GTP:ATP:CTP:UTP (8:1:1:1:1 mM) was used.\(^{23}\) The RNA was purified by phenol/chloroform extraction and ethanol precipitation. ATTO 488 was then conjugated onto RNA via the 5' thiol group following a reported method.\(^{24}\) 5'-GMPS primed pre-miR-155 (10 µg) was dissolved in PBS (1 mL, pH 8.3), followed by the addition of ATTO 488-Iodoacetamide for pre-miR-155 and ATTO 590-Iodoacetamide for pre-miR-21 (1.3 equiv) (ATTO-TEC). The reaction was kept in dark at 37 °C for 2 h. The labeled RNA was purified by phenol/chloroform extraction and ethanol precipitation.

Cell culture

HEK293T and MCF-7 cells were cultured in DMEM medium (Gibco) without antibiotics, supplemented with 10% FBS and 2 mM GlutaMAX (Life Technologies) at 37 °C in a humidified atmosphere containing 5% CO\(_2\).

Dicer cleavage inhibition assay

\(^{32}\)P-labeled pre-miR-155 was prepared as described earlier. A 10 µL of the reaction mixture was made by incubating \(^{32}\)P-labeled pre-miR-155 (1 µL, ~20 ng) with Dicer enzyme (Gen-Lantis) (1 µL) and various concentrations of 24 in buffer (HEPES 24 mM, NaCl 200 mM, EDTA 0.04 mM, MgCl\(_2\) 2.5 mM, ATP 1 mM, pH 7.5) at 37 °C for 2.5 h. The reaction was stopped by boiling with equal volume of Gel Loading Buffer II (ThermoFisher Scientific) for 5 min. The non-cleaved pre-miR-155 and the processed miR-155 were resolved by 18% denaturing polyacrylamide gel. The gel
was imaged with phosphor imager and analyzed by quantity one software (Bio-
rad).

**MiR-155 inhibition in cell**

MCF-7 cell was used for studying the activity of 24 in inhibiting endogenous miR-
155 in cell. The cells were plated in 24-well plates (500 µL of medium per well) and
grown overnight to ~60% confluency. The cells were then treated with 24 (30 µM),
DMSO (0.2% v/v) or anti-miR-155 (30 nM, transfected as described above)
(Integrated DNA Technologies). The cell cultural medium was replaced with fresh
one containing the corresponding small molecule every 24 h. The cells were
harvested after 4-day incubation for the following RT-qPCR, Western blotting, and
flow cytometry analysis.

**RNA extraction (RNAseq sample preparation)**

Total RNA was extracted using miRNeasy Mini Kit (Qiagen) per the manufacturer's
protocol for both RT-qPCR and RNAseq. MiRNA reverse transcription reactions
were completed using a Taqman MicroRNA RT Kit (Applied Biosystems) per the
manufacturer’s protocol analyzed by nanodrop and sent for sequencing.
4.5 References


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