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Aryl amide small-molecule inhibitors of microRNA miR-21 function

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ABSTRACT

MicroRNAs (miRNAs) are single stranded RNA molecules of ~22 nucleotides that negatively regulate gene expression. MiRNAs are involved in fundamental cellular processes, such as development, differentiation, proliferation, and survival. MiRNA misregulation has been linked to various human diseases, most notably cancer. MicroRNA-21 (miR-21), a well-established oncomiR, is significantly overexpressed in many types of human cancers, thus rendering miR-21 a potential therapeutic target. Using a luciferase-based reporter assay under the control of miR-21 expression, a high-throughput screen of >300,000 compounds led to the discovery of a new aryl amide class of small-molecule miR-21 inhibitors. Structure–activity relationship (SAR) studies resulted in the development of four aryl amide derivatives as potent and selective miR-21 inhibitors. The intracellular levels of various miRNAs in HeLa cells were analyzed by qRT-PCR revealing specificity for miR-21 inhibition over other miRNAs. Additionally, preliminary mechanism of action studies propose a different mode of action compared to previously reported miR-21 inhibitors, thus affording a new chemical probe for future studies.

MicroRNAs (miRNAs) are small, single stranded non-coding RNAs that play a critical role in the post-transcriptional regulation of gene expression.[1] MiRNAs are processed via a dedicated pathway: the miRNA genes are transcribed by RNA polymerase II, delivering primary miRNAs, which are subsequently processed by Drosha to afford precursor miRNAs. These precursors are then exported to the cytoplasm where they are digested by the Dicer enzyme and loaded into the RNA-induced silencing complex (RISC). Association of the miRNA-loaded RISC with target mRNA induces sequence-specific translational repression or mRNA degradation.[2] Over 2500 miRNAs have been discovered in humans[3] and involvement, in part, in the regulation of more than 60% of protein-coding genes has been proposed.[4] Not surprisingly, miRNAs are key players in the control of essential processes such as cell proliferation, cell differentiation, and apoptosis.[5] As a result, misregulation of miRNA levels has been implicated in contributing to many human diseases, most importantly cancer.[1]

The miRNA miR-21 is one of the most extensively studied miRNAs to date. It was one of the first miRNAs identified in the human genome and is the only known miRNA to be overexpressed in nearly all types of human cancers.[6] Its oncogenic role was first discovered by miRNA profiling in glioblastoma tumor samples obtained from cancer patients.[7] Additional large-scale profiling investigations have identified that the overexpression of miR-21 can represent up to 25% of the total cellular miRNA content in cancer cells.[8] Furthermore, the oncogenic properties of miR-21 were investigated in an in vivo pre-B-cell lymphoma mouse model in which down-regulation of miR-21 demonstrated significant therapeutic potential and highlighted the oncogene addiction of tumor cells to miR-21.[9] The aberrant expression of miR-21 in many cancers has spurred the investigation of how miR-21 expression is regulated and how it can be efficiently inhibited with chemical tools.

Oligonucleotide-based reagents such as anti-miRNA oligonucleotides provide the most direct route to the inhibition of miRNA function. Anti-miRNA oligonucleotides are synthetic, chemically modified oligonucleotides with the exact complementary sequence of the miRNA of interest. Their specificity and efficiency make them excellent tools, however, oligonucleotides face challenges to therapeutic use. Due to their generally poor cellular delivery,[10] and the occurrence of off-target effects,[11] such as immune response stimulation and liver toxicity,[12] oligonucleotide-based cancer therapeutics have yet to reach the clinic. Small-molecule inhibitors of miRNA function have potential to overcome these barriers due to their stability in vivo, reversibility, cost effective production, ease of delivery, and improved pharmacokinetics.[10,13] Additionally, unlike current oligomer-based tools, which regulate miRNA function through a direct interaction with mature miRNA, small-molecules can perturb miRNA function at various steps of miRNA biogenesis and processing. For example, small-molecules could be used to modulate upstream pathways

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involved in the transcription of miRNAs, interfere with the miRNA maturation process, or inhibit incorporation of the mature miRNA into RISC.[14] Thus, the discovery of small-molecule modulators of miRNA function has gained significant momentum in recent years.[15–24]

Previously, we identified a selective small-molecule inhibitor of miR-21 from a pilot screen in HeLa cells using a luciferase-based reporter assay that places luciferase expression under the regulation of miR-21 activity.[15] In order to identify additional, structurally diverse small molecule miR-21 inhibitors, an extensive high-throughput screen of 333,519 compounds was performed (pubchem AID 2289), which delivered 3282 hits (1% hit rate). Subsequently, 124 of the top ranking small molecules identified in the primary assay were submitted to secondary screens to evaluate their selectivity, as well as their specificity. The compounds were tested in a cell-based assay expressing a reporter construct targeting miR-30a (pubchem AID 2507). Compounds active in both the miR-21 and miR-30a assays were disregarded as they were not considered selective for miR-21 inhibition, but rather may be general miRNA modulators or non-specifically target firefly luciferase. Additionally, the initial hits were subjected to an in vitro firefly luciferase assay using recombinant firefly luciferase enzyme (pubchem AID 493175). Compounds identified as firefly luciferase inhibitors were disregarded as false positives (see detailed discussion below). Ultimately, 58 small molecules were confirmed as inhibitors of miR-21. After collecting dose–response data and qRT-PCR results on the down-regulation of miR-21 levels, the aryl amide **1** was selected for follow-up SAR studies.

Following re-synthesis and confirmation of **1**, structure–activity relationship studies were conducted through the synthesis and testing of analogs (Fig. 1). Replacing the bulky piperidine moiety with a smaller acetyl (**2**) or methoxy (**3**) group resulted in favorable 47% and 65% increases in activity, respectively. Further modification of the *meta* position to a hydroxyl group (**4**) showed a more modest 27% increase in activity over the parent compound **1**, while removal of the *meta* substituent altogether (**5**) completely abolished activity. Interestingly, modification of the *para* or *meta* positions to hydroxyl groups (**6**, **7**) or removal of both substitutions (**8**) resulted in very little change to the activity, while the 1,3-disubstituted hydroxyl derivative (**9**) displayed a very promising 86% increase in activity. Modification of 4 from a cyclohexene to a cyclohexane (**10**) resulted in a 57% increase in activity relative to **1**, while both its benzene derivative (**11**) or loss of the ring entirely (**12**) resulted in complete loss of activity. Surprisingly, modification of the *p*-methoxy to an ethyl ether (**13**), propyl ether (**14**), benzyl ether (**15**), or amine (**16**) rescued activity. Retaining the *p*-methoxy while extending the aliphatic linker by an additional carbon (**17**) yielded a 58% increase in activity relative to the parent compound **1**. Based on the identification of **17**, several new analogs were synthesized that modified the benzene ring and its aliphatic linker. Extending or shortening the linker by an additional carbon (**18**, **19**) resulted in complete loss in activity. Modification of the benzene ring in **19** to a 2-pyridine (**20**) also resulted in a loss in activity. Introduction of a naphthalene (**21**) rescued partial activity, as did removing an additional carbon from the linker (**22**). Completely removing the aliphatic linker (**23**) resulted in a drastic 98% increase in activity over **1**. Replacement of the benzene ring with a naphthalene (**24**), a propylene (**25**), or the free amide (**26**) only reduced the activity. Removal of the *p*-methoxy (**27**) from **23** also resulted in a loss in activity, but introduction of an additional *p*-methoxy on the benzene ring (**28**) resulted in a derivative with a 152% increase in activity over the original hit compound **1**. Based on the results from the SAR investigation, four of the most potent aryl amides **3**, **9**, **23**, and **28** were further investigated.

While highly sensitive, the use of a luciferase-based assay in a HTS requires validation of hit compounds through secondary assays. In recent literature, a concern for the presence of firefly luciferase inhibitors in large compound libraries and the consequential appearance of false positives has been reported.[25] The firefly luciferase enzyme is sensitive to ligand-based stabilization by small molecule inhibitors; therefore in a cell-based assay, treatment with an inhibitor can lead to an increase in luciferase signal after cell lysis and assay readout. Conversely, biochemical evaluation of the same inhibitors causes a decrease in luciferase signal.[25] In particular, compound **28** has been previously identified as a firefly luciferase inhibitor in a biochemical qHTS (PubChem AID 411). In order to confirm that the miR-21 inhibitors are not in fact inhibiting luciferase, an in vitro luciferase assay was employed. Treatment of firefly luciferase enzyme with **3**, **9**, **23**, or **28** at 10 μM showed no effect on luciferase activity validating that these compounds do not inhibit luciferase enzyme (SI Fig. 1).

To investigate each inhibitor's selectivity for miR-21, compounds **3**, **9**, **24**, and **28** were tested at 10 μM in our previously described Huh7-psiCHECK-miR122 reporter cell line, which expresses *Renilla* luciferase under the control of miR-122 activity.[16] None of the four inhibitors displayed any effect on luciferase expression in this secondary assay, while showing a greater than 100% increase in luciferase expression in the HeLa-miR21-Luc cell line (Fig. 2A). This provides evidence that these compounds display (some level of) specificity to miR-21 and are not general inhibitors of the miRNA pathway. Furthermore, satisfactory dose response curves were generated for each compound in the HeLa-miR21-Luc assay revealing EC_{50} values of 10.8 μM, 6.1 μM, 2.3 μM, and 0.86 μM, respectively (Fig. 2B).

Inhibitors **3**, **9**, **23**, and **28** were then analyzed by quantitative RT-PCR to measure their direct effect on miR-21, miR-125b, miR-17-5p, and miR-222 expression levels in HeLa cells. Cells were incubated with **3**, **9**, **23**, or **28** at 10 μM for 48 h, the total RNA was isolated (miRNeasy kit, Qiagen), and the quantification was performed with the corresponding TaqMan microRNA assays (Life Technologies). While **3** showed only a small 17% reduction in miR-21 expression, the inhibitors **9**, **23**, and **28** showed more significant reductions of 42%, 43%, and 61%, respectively (Fig. 3A). These values correlate well with their respective EC_{50} values determined in the HeLa-miR21-Luc assay. Additionally, all four inhibitors showed little or no effect on miR-125b, miR-17-5p, or miR-222 levels, providing additional support that these compounds may be specific to miR-21.

To explore a possible mode of action for the inhibitors, RT-PCR was used to specifically quantify the expression levels of primary-miR-21 in HeLa cells. Following treatment with **3**, **9**, **23**, and **28**, little to no effect on primary-miR-21 levels was observed, suggesting that these inhibitors may act downstream of transcription of the miR-21 gene (Fig. 3B). This is in contrast to previously discovered small molecule inhibitors of miRNA function and may lead to fundamentally new chemical probes to interrogate the miRNA pathway.[15,16,20,21] To further confirm this, the endogenous miR-21 promoter was cloned upstream of a luciferase gene in the pGL4 construct (Promega), thereby placing the reporter under control of the miR-21 promoter. Transfection of the pGL4-miR21P plasmid into HeLa cells resulted in a >3000-fold increase in luciferase signal compared to the parent pGL4-empty vector (Fig. 4A). Next, HeLa cells transfected with the pGL4-miR21P vector were treated with DMSO or inhibitors **3**, **9**, **23**, or **28**, followed by a luciferase assay to determine reporter gene expression. As expected, treatment with the inhibitors had no effect on the reporter gene and thereby the miR-21 promoter (Fig. 4B), further supporting that these inhibitors do not effect the transcription of miR-21, but may rather inhibit the miR-21 maturation pathway. Although quite unlikely due to the relatively small and planar core structures of the arylamide miR-21 inhibitors, another possibility would be that these compounds inhibit miR-21 by direct interaction with the miRNA.

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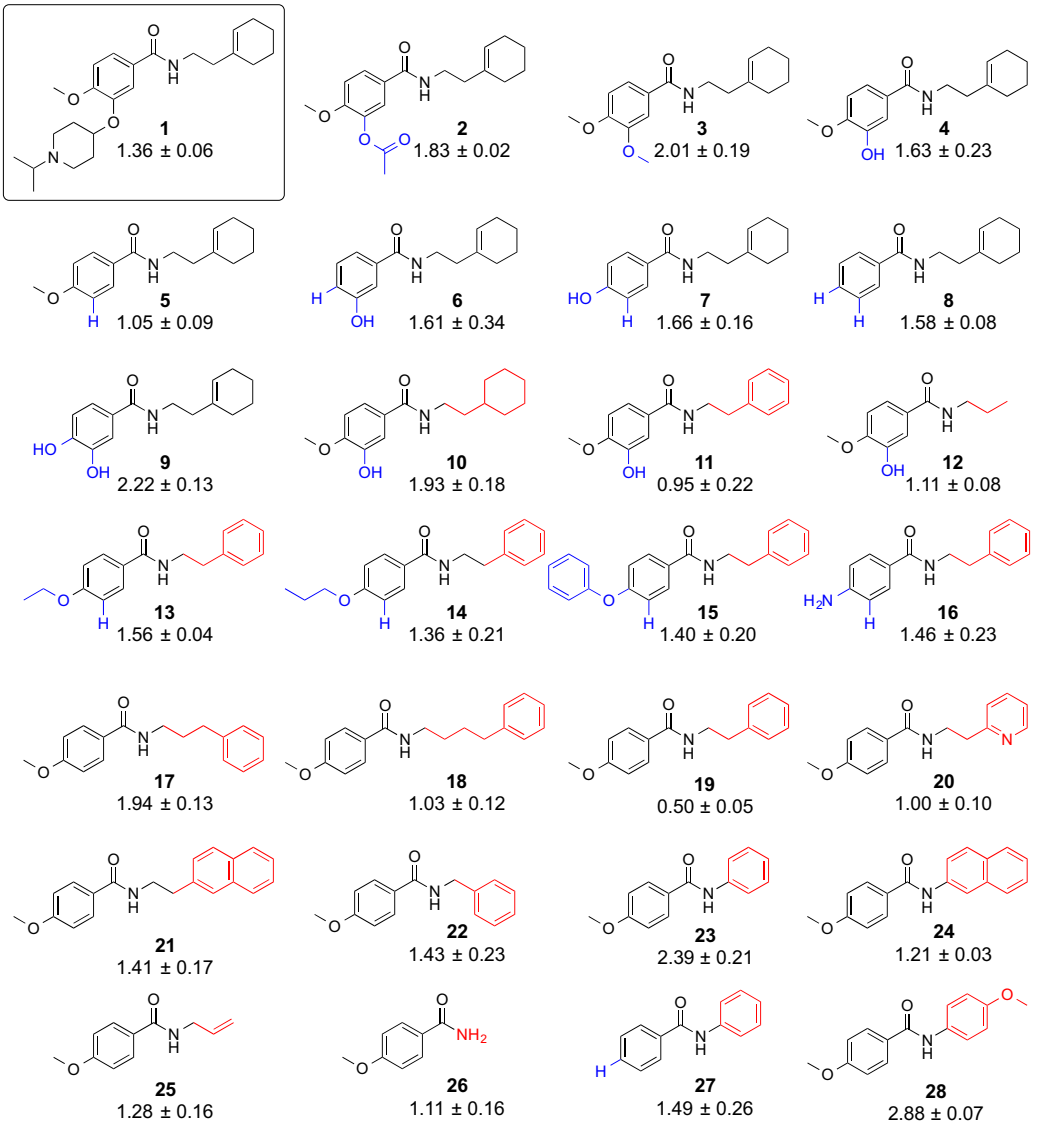

Figure 1. Aryl amide high-throughput screening hit **1** and structure–activity relationship studies. Values represent fold-change in luciferase signal normalized to cell viability and relative to DMSO (negative control). Errors represent standard deviations of three independent experiments.

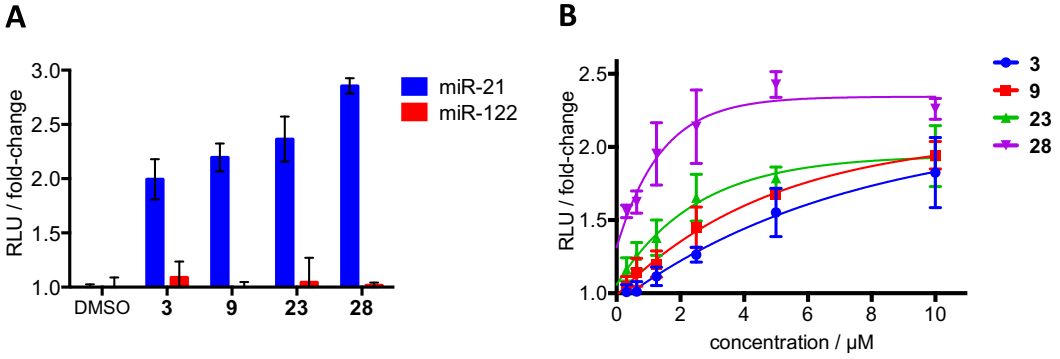

Figure 2. (A) Inhibitors **3**, **9**, **23**, and **28** (10 μM) effect on luciferase expression in the HeLa-miR21-Luc and Huh7-psiCHECK-miR122 stable cell lines. (B) Luciferase dose response curves for inhibitors **3**, **9**, **23**, and **28** in the HeLa-miR21-Luc cell line. All data was normalized to the DMSO (negative) control and errors represent standard deviations of three independent experiments.

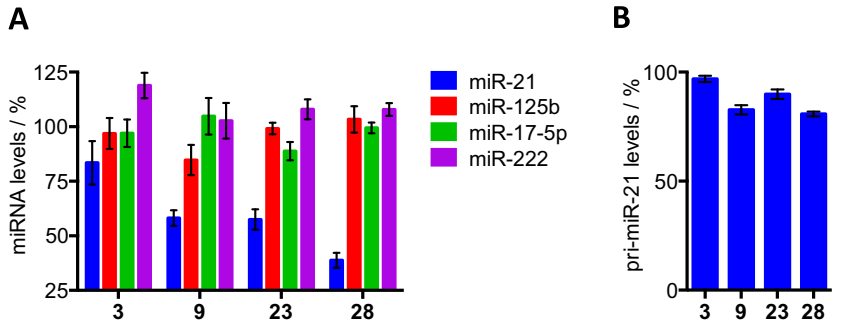

Figure 3. (A) RT-PCR quantification of miR-21, miR-125b, miR-17-5p, and miR-222 in HeLa cells treated with inhibitors **3**, **9**, **23**, or **28** (10 μM) for 48 h. (B) RT-PCR quantification of primary-miR-21 in HeLa cells treated under the same conditions. qRT-PCR analysis conducted using the $2^{\Delta\Delta C_t}$ method with RNU19 as an internal standard. All data was normalized to DMSO-treated cells (negative control) and error bars represent standard deviations from three independent experiments.

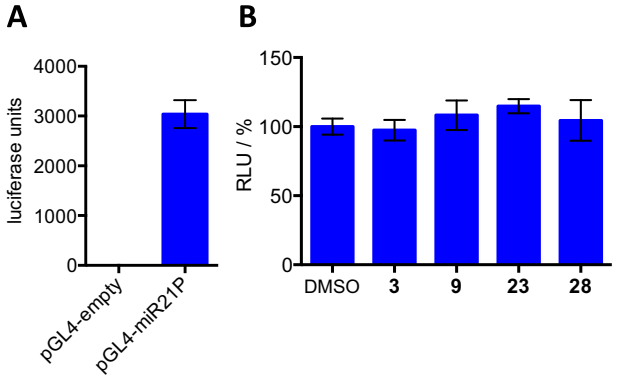

Figure 4. (A) Transfection of pGL4-empty and pGL4-miR21P into HeLa cells. (B) Transfection of pGL4-miR21P into HeLa cells treated with inhibitors **3**, **9**, **23**, or **28** (10 μM). Data was normalized to DMSO (negative control) treatment. Error bars represent standard deviations from three independent experiments.

Additional studies are under way to further elucidate the mode of action of this new class of miRNA inhibitors.

In summary, a high-throughput screen of >300,000 compounds led to the identification of a new class of small-molecule inhibitors of miR-21. Subsequent structure–activity relationship studies produced four potent inhibitors that exhibit selectivity for miR-21 over other miRNAs. Furthermore, these compounds appear to inhibit miR-21 in a new mode of action compared to existing molecules. Since they act downstream of miRNA transcription, they may provide new probes to study the miRNA maturation pathway for miR-21. Further studies are ongoing to investigate the mechanism of action of these probes, as well as to evaluate their effectiveness as therapeutic agents for the treatment of cancer.

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Supplementary data

Supplementary data (detailed experimental procedures and characterization data) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.07.016.

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