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Small-Molecule Inhibitors of MicroRNA miR-21 Function**

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MicroRNAs (miRNAs) have recently emerged as an important class of gene regulators, and their misregulation has been linked to a variety of cancers. Small-molecule inhibitors of miRNAs would be important tools for the elucidation of the detailed mechanisms of miRNA function and should serve as lead structures for the development of new therapeutic agents. We report a cellular screen for miRNA-pathway inhibitors and the first small-molecule modifiers of miRNA function.

miRNAs are single-stranded noncoding RNAs of 21–23 nucleotides. They form a novel class of gene regulators that function by binding to the 3′ untranslated region of target messenger RNA (mRNA) molecules. This interaction leads to either suppression of translation, or acceleration of the degradation of the mRNA.[1] The majority of miRNA molecules are initially transcribed as primary miRNAs (pri-miRNAs).[2] Pri-miRNAs are processed further in the nucleus by the enzyme Drosha, which transforms pri-miRNAs into shorter stem-loop-structured, double-stranded RNA molecules called precursor miRNAs (pre-miRNAs).[3] Pre-miRNAs are then transported from the nucleus to the cytoplasm, where they are processed by Dicer into mature miRNAs.[4] Mature miRNAs enter the effector complex, known as the RNA-induced silencing complex (RISC), to then target single-stranded complementary mRNAs (see Figure 1 in the Supporting Information).[5] It is estimated that miRNAs are involved in the regulation of about 30% of all genes and almost every genetic pathway.[6] Moreover, recent evidence suggests that they can function as oncogenes and tumor suppressors.[7,8] Thus, small-molecule regulation of misregulated miRNAs has the potential to become a new area of therapeutics. Until now, specific miRNA inhibition has only been observed with antisense nucleic acids.[9]

We developed an assay for small-molecule inhibitors of miRNA function and discovered potentially specific miRNA-pathway inhibitors. Although inhibitors of the siRNA (small interfering RNA) pathway have been identified,[10] to our knowledge no small-molecule inhibitors of the miRNA pathway have been reported. We selected miR-21 as the target miRNA as a result of its documented function as an antiapoptotic factor in cancer cells and its elevated levels in various cancers, such as breast, ovarian, and lung cancer, as well as glioblastomas.[7,11] Lentiviral reporter constructs for miRNA activity were assembled by introducing the complementary sequences to those of mature miR-21, the specificity control miR-30, or a negative-control linker sequence (a site with no detectable recognition by natural miRNAs) downstream of a luciferase reporter gene (see Figure 2 in the Supporting Information). These plasmids serve as sensors to detect the presence of specific mature miRNA molecules (Figure 1).

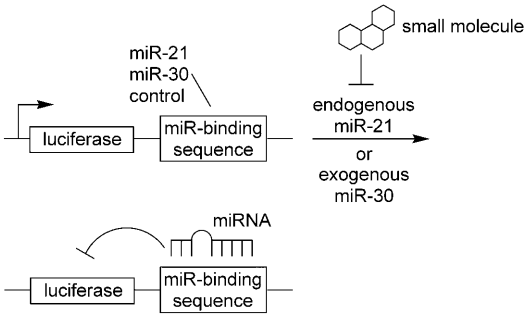

Figure 1. Luciferase expression under the control of an miRNA-binding sequence in the 3′ untranslated region (3′ UTR) provides an efficient miRNA assay. Endogenous miR-21 (HeLa cells) or exogenous miR-30 downregulate luciferase activity when paired with their specific binding sequence.

The reporter constructs were introduced stably into HeLa cells, which express high levels of miR-21 but relatively low levels of miR-30.[12] To test the miRNA specificity of the reporter system, cells that contained both the luciferase miR-30A (Luc-miR-30A) reporter construct and a construct that expresses exogenous primary miR-30 were assayed. The luciferase signal of these cells was greatly diminished relative to that of cells with a mismatched Luc-miR-30A reporter/miR-21 combination (see Figure 3 in the Supporting Information). Thus, the Luc-miR-21 and Luc-miR-30A reporters

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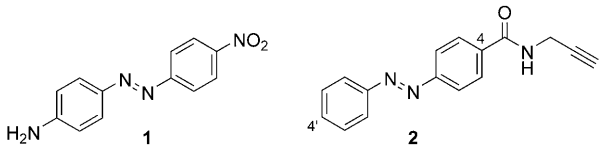
are specific and react only to miR-21 and miR-30, respectively. The ability to detect endogenous miR-21 was proven by the fact that the introduction of the Luc-miR-21 reporter into HeLa cells led to a 90% decrease in the intensity of the luciferase signal relative to that of the control luciferase-linker construct, a result that indicates the high level of endogenous miR-21 expression in HeLa cells (see Figure 4 in the Supporting Information). As expected, the introduction of the miR-30A reporter led to only a modest decrease in the intensity of the luciferase signal, as HeLa cells express relatively low levels of endogenous miR-30.

Subsequently, a primary screen of more than 1000 compounds from our own compound collection and the Library of Pharmacologically Active Compounds (Sigma–Aldrich) was conducted at a compound concentration of 10 μM. An initial hit compound, diazobenzene **1**, produced a 251% increase in the intensity of the luciferase signal relative



to that of untreated cells (the dimethyl sulfoxide (DMSO) control had no effect on the luciferase signal; see Figure 5 in the Supporting Information). Through several rounds of screening and structural modification, a preliminary structure–activity relationship was developed (see Figure 6 in the Supporting Information). Acylation and alkylation of the amino group in **1** led to diminished activities. However, the screening of a wide range of structurally related molecules with the azobenzene core uncovered the highly active compound **2**, for which a fivefold increase in the luciferase signal was observed at a concentration of 10 μM (Figures 2 and 3a). The modification of **2** through the introduction of an amino or nitro group at the 4′-position led to a 12 and 64%

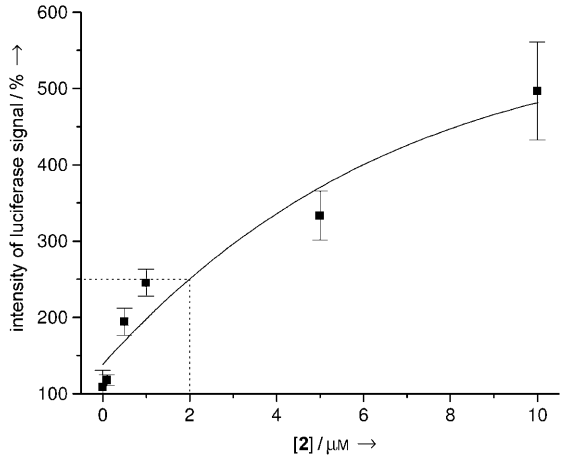


Figure 2. Dose-response curve for **2**. The results reveal an EC_{50} value of 2 μM and an increase of approximately 500% in the intensity of the luciferase signal at 10 μM. The error bars indicate the standard deviation determined from three independent measurements.

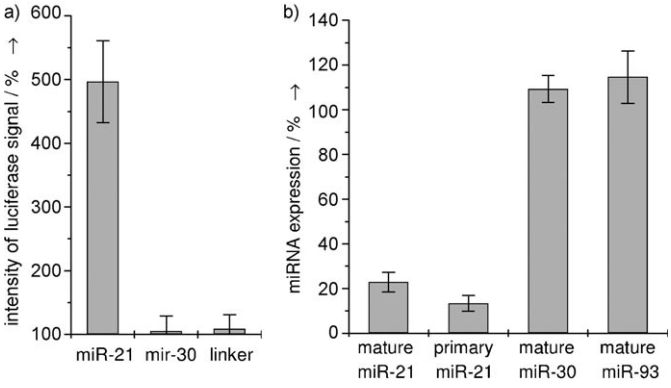


Figure 3. a) Change in the luciferase signal of cells treated with **2** (10 μM) relative to a DMSO control. b) Levels of mature or primary miRNA in cells treated with **2** (10 μM) relative to a DMSO control, as determined by quantitative RT-PCR. The error bars indicate the standard deviation determined from three independent measurements.

decrease in activity, respectively. A substantial loss of activity (24–53%) was observed with other amide substituents, with the exception of allyl and propyl groups, which led to 11 and 16% lower activity, respectively. An exchange of the amide for a sulfonamide functionality delivered compounds with no activity. Interestingly, the activity of the styrene analogue of **2** was 40% lower than that of **2**. Compound **2** is the most effective small-molecule inhibitor of microRNA miR-21 function discovered so far. It induces a 485% increase in the intensity of the luciferase reporter signal at a concentration of 10 μM. The diazobenzene **2** does not display cytotoxic effects at this concentration, as determined by an MTT assay (MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; data not shown). Dose-response studies carried out at concentrations of 0–10 μM revealed a concentration dependence of the luciferase signal with an EC_{50} value of 2 μM (Figure 2).

Several experiments were conducted to investigate the mode of action of the inhibitor **2**. The compound does not affect the luciferase signal in HeLa cells that express the Luc-linker control sequence (Figure 3a). This result indicates that **2** only increases the intensity of the luciferase signal through inhibition of the miRNA pathway. Furthermore, HeLa cells that express both the miR-30 luciferase reporter construct and miR-30 were treated with **2**. In this case, no increase in the intensity of the luciferase signal was detected (Figure 3a), which indicates that **2** is potentially specific towards miR-21 and does not have a general effect on the common miRNA pathway.

The specificity of **2** for the inhibition of miR-21 function was further validated by measuring intracellular miRNA levels by quantitative RT-PCR (RT = reverse transcription; Figure 3b). We found that levels of the stably expressed exogenous mature miR-30, endogenous mature miR-93, and endogenous non-miRNA genes, such as E-chaderin, ID1, RAP1A, and fibronectin, are not diminished upon treatment with **2** (Figure 3b; see Figure 7 in the Supporting Information). Gratifyingly, miR-21 expression is decreased by 78% relative to expression with the DMSO control in HeLa cells. Quantitative RT-PCR experiments with primers specific for

the primary miR-21 (pri-miR-21) sequence but not mature or precursor miR-21 revealed that the pri-miR-21 levels in cells treated with **2** were decreased by 87% (Figure 3b). Similar observations were also made for three other cell lines, MCF-7, A172, and MDA-MB-231, which express miR-21 endogenously (see Figures 7 and 8 in the Supporting Information). These results strongly suggest that compound **2** is an inhibitor that targets the transcription of the miR-21 gene into pri-miR-21, but not downstream processes of the common miRNA pathway.

In summary, we have developed a method to identify inhibitors of the miRNA pathway in live cells, specifically of miR-21, an important antiapoptotic factor in several cancers. A screening of more than 1000 small organic molecules, followed by structure–activity-relationship analysis of an initial hit, uncovered the azobenzene **2** as a specific and efficient inhibitor of miR-21 expression. Research on miRNAs is still in its infancy, and the biogenesis of many miRNAs (including miR-21) is not fully understood. Therefore, specific inhibitors (such as **2**) of the miRNA pathway will be unique tools for the investigation of miRNAs and their involvement in various types of diseases.

Experimental Section

Screening protocol: HeLa cells were cultured in the Dulbecco Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), and 2500 HeLa cells containing the Luc-miR-21 construct were plated into each well of a 384-well plate. The plates were incubated overnight at 37°C with 5% CO_2. Compounds were added to a final concentration of 10 μM. Luciferase signals were measured 48 h after treatment with the compounds by using a Bright-Glo luciferase assay kit (Promega) according to the protocol described by the manufacturer. The data of three independent experiments were averaged.

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