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A Molecular-Beacon-Based Screen for Small Molecule Inhibitors of miRNA Maturation

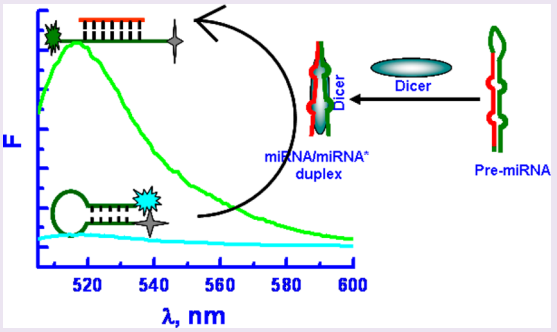
Debojit Bose,[†] Gopal Gunanathan Jayaraj,[†] Santosh Kumar,[†] and Souvik Maiti*[,†,‡]

[†]Proteomics and Structural Biology Unit CSIR-Institute of Genomics and Integrative Biology, Mall Road, Delhi 110007, India

[‡]CSIR-National Chemical Laboratory, Dr. Homi Bhabha Road, Pune 411008, India

Supporting Information

ABSTRACT: miRNAs are small non-coding RNAs that regulate about 60% of mammalian genes by modulating their transcript levels. Network scale studies of miRNA-mediated regulatory circuits demonstrate the central importance of this class of small RNA in the maintenance of biological robustness. More recently, several reports have described the deregulation of numerous miRNA to be causally associated with many diseases, including cancer. These studies have highlighted the potential for development of therapeutic modalities against miRNA. Previous screening protocols, for small molecules targeting miRNA function, are either costly or technically too complex to be applied in a high-throughput manner in standard chemical laboratories. We describe a simple *in vitro* screening method using a DNA-based molecular beacon that overcomes the limitations associated with earlier screens. We used this method to identify inhibitors of miR-27a function from a library of 14 aminoglycosides as a pilot study. Inhibitory molecules identified were further scrutinized to identify the validity of screen. With this proof of concept we illustrate the utility of a scalable molecular-beacon-based screening strategy for miRNA inhibitors.



miRNAs are one of the best studied classes of small non-coding RNA, having physiological as well as pathological functions. Mature miRNAs are ∼23 nucleotides in length and negatively regulate gene expression by binding to complementary sites in target mRNA.[1] They are transcribed as long precursor transcripts that through a series of enzymatic steps are converted to the mature cytoplasmic miRNA.[2] miRNAs are nodal regulators of many biological processes that include development, stress response, immunity, and induction and maintenance of pluripotency.[3−6] MiRNAs have been implicated in various pathological conditions including cancer, cardiovascular disorders, diabetes, and neurological disorders.[7−9] Modulation of miRNA levels has been demonstrated as a viable strategy for a number of diseases.[10] Classically the well-known miRNA modulators, currently used in preclinical and clinical studies, include antisense oligonucleotides (anti-miRs), antagomirs, miRNA sponges, and DNAzymes.[11−15] However, the inefficient delivery and suboptimal pharmacodynamic/pharmacokinetic properties are major hurdles in oligonucleotide-based therapeutics highlighting the importance and need for small-molecule-based intervention strategies.[16]

Secondary structures are abundant in RNA molecules. The RNA duplex is of he A-form, and the major group is only 4 Å wide, which renders sterical hindrance in small molecule binding. Regions with a perturbation of the A-form helix are optimal for RNA targeting. Such perturbations create various classes of secondary structures, such as hairpin loops, internal loops, and bulged regions. These perturbations, induced by unor mispaired bases, widen the major groove, providing a surface-exposed binding pocket for proteins and small molecules and making them good drug targets.[17,18] Recently small molecules have been identified that modulate miRNA expression.[19−25] As mentioned, miRNA maturation involves a number of precursor forms that are rich in secondary structures such as stem loops and bulges. The secondary structures, essential for their biogenesis, bind to ligands selectively and thereby allow modulation of expression. One of the earliest attempts in this regard was the development of an *in vitro* assay for screening for compounds that block the dicing activity of Dicer.[19] More recently, cell-based assays have been used to identify small molecule modifiers of the miR-21 and miR-122.[20,21] Our group has identified streptomycin from an aminoglycoside screen and shown that this selective scaffold ligand interferes with Dicer-mediated processing of pre-miR-21, inhibiting its maturation.[24] Although these studies have applied high-throughput cell-based assays to identify miRNA modulators and biological outputs, such assays are relatively time-consuming and do not provide information on the mechanistic details of the modulation. On the other hand, *in vitro* cell-free systems have high throughput and can reveal clues about the nature of ligand-miRNA interaction, thus making them amenable to drug discovery platforms.

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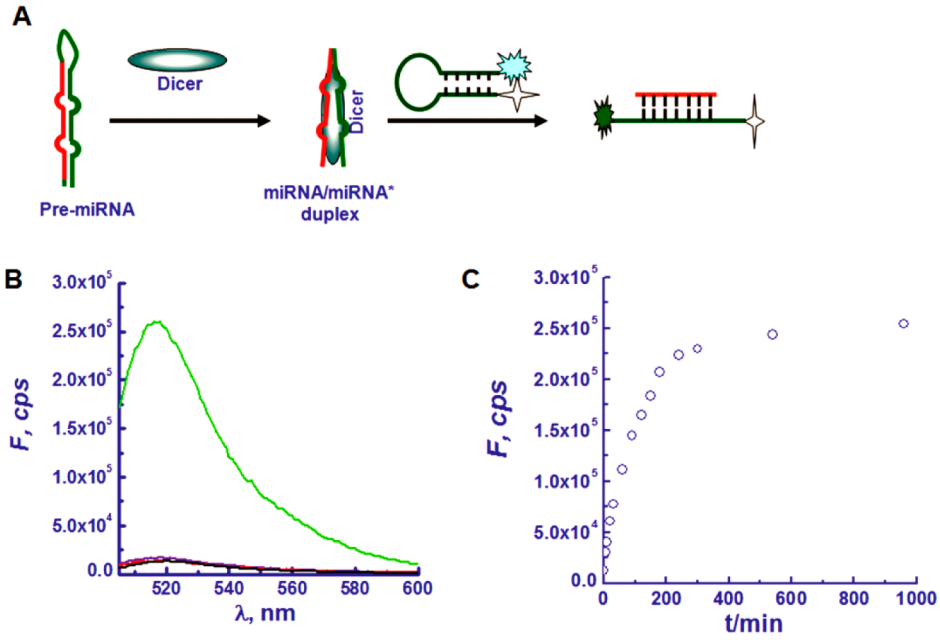

Figure 1. Mechanism of action of molecular beacon: schematic and fluorescence study. (A) Schematic representation of mechanism of action of the molecular beacon. The beacon contains a stem loop structure with a fluorophore and a dark quencher at the two ends, where the loop sequence is exactly complementary to miRNA (anti-miR). In the presence of the mature miRNA of interest only the beacon will open up and hybridize with it, causing an increase in fluorescence signal. (B) Beacon only (black line), beacon with pre-miR-27a (violet line), and beacon with Dicer (red line) do not show any detectable increase in fluorescence intensity, proving that the beacon does not open up in the presence of pre-miRNA or Dicer. In the presence of the beacon, pre-miR-27a, and Dicer, a huge increase in fluorescence intensity was seen, confirming that the beacon opens up and hybridizes with mature miRNA. (C) Time-dependent Dicer kinetics: pre-miR-27a with the molecular beacon was incubated, followed by the addition of Dicer. Fluorescence spectra were taken at regular time intervals, starting from 5 min till 16 h. Saturation in fluorescence signal increment was found after around 4 h.

Here we report the development of a novel method for high-throughput screening of miRNA modulators based on DNA-molecular beacons. miRNA biogenesis is a tightly regulated process in which pre-miRNAs are cleaved by Dicer to produce mature miRNAs. The mature miRNAs associate with RISC complex and bind to the 3′ UTR of target mRNAs causing translational repression, cleavage, and/or destabilization. Hence the approach was to screen for compounds that could interfere with miRNA function by inhibiting Dicer-mediated miRNA maturation. We chose miR-27a as a model miRNA as it is reported to be overexpressed in a number of different cancers.[26−29] Molecular beacons have recently been used in miRNA quantification as well as miRNA expression profiling.[30,31] Although beacon-based screening of miRNA inhibitors has already been described earlier,[19] there are some drawbacks associated with the study. The authors used a pre-miRNA-based molecular beacon, where a fluorophore molecule is attached at the 5′ end of the pre-miRNA and a fluorescence quencher at the 3′ end. Recent reports however suggest that human Dicer depends on both the 5′ end and 3′ end of the pre-miRNA for proper cleavage and miRNA maturation.[32−35] Thus addition of fluorophore and quencher might affect the binding and activity of Dicer enzyme, thereby obscuring the results. Although the authors argue that Dicer also binds to perfect dsRNA ends, that cleavage mechanism is not used in pre-miRNA processing inside the cell. Thus their system may not truly mimic the Dicer processing of pre-miRNA and hence may not be a suitable context to identify small molecule inhibitors of pre-miRNA processing. We address these drawbacks by making use of a DNA molecular beacon (with a 5′ fluorophore and 3′ quencher) that is independent of the pre-miRNA and is complementary to the mature miRNA produced after cleavage by Dicer.

Using our beacon-based assay, as a proof of principle, we screened 14 commercially available aminoglycosides and found that a set of them inhibit Dicer-catalyzed miR-27a maturation. The effect of these small molecules was further validated by a cell-based assay, followed further by biological studies.

RESULTS AND DISCUSSION

Design of DNA-Beacon-Based Screening System. The design of the DNA beacon (hereafter referred to as anti-miR-27a beacon) is represented in Figure 1A. The anti-miR-27a beacon was constructed by placing 5′ FAM and 3′ BHQ1 quencher at the ends of the anti-miR-27a stem loop. The beacon loop is a perfect complement of the mature miR-27a. In the natural conformation only low levels of fluorescence would be observed due to close proximity of fluorophore and quencher. In pre-miR-27a, the mature strand is in base pairing with the passenger strand, forming a stem loop structure that is more thermodynamically stable. Hence the beacon will not be able to open up and there will be no fluorescence. However, the dicing and helicase activity of Dicer will result in the formation of mature miR-27a, which in turn will hybridize with the loop of anti-miR-27a beacon. This will result in the opening of the beacon, leading to increase in fluorescence. Conversely, in the presence of a small molecule that blocks Dicer processing by binding to pre-miR27a, the beacon is restricted to the closed native conformation and thus a reduced fluorescence should be observed (Figure 1A).

Beacon-Based Small Molecule Screening. Prior to screening for small molecule candidates using the molecular

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beacon, we sought to explore the specificity of the anti-miR-27a beacon toward mature miR-27a. To do so, the beacon was incubated with pre-miR-27a or Dicer, respectively. No increase of fluorescence intensity was observed (Figure 1B). This ruled out the possibility of nonspecific hybridization of beacon with pre-miRNA as well as the opening up or cleavage of beacon in the presence of Dicer. Conversely, preincubation of beacon and pre-miR-27a followed by the addition of Dicer showed increase in fluorescence signal in a time-dependent manner. The dicing kinetics was measured in a time scale from 5 min to 16 h, and a saturation of fluorescence signal increment was observed after 4 h (Figure 1C). Thus we selected 4 h as a suitable measurement time for the screens described hereafter. Any small molecule having the potential to bind to pre-miR-27a and/or Dicer and thereby hinder Dicer processing will affect the increment of fluorescence signal.

This strategy was used to identify small molecule inhibitors of miR-27a. Aminoglycosides are known to bind to RNA secondary structural motifs, such as stem loop junctions, bulges, and hairpin and internal loops. Different aminoglycosides have differential preferences for RNA secondary structure motifs.[36] In the miRNA biogenesis pathway, precursor miRNAs fold into stem loop structures with internal bulges, and these structural features are essential for Dicer-dependent miRNA maturation. These structural features of pre-miRNA make them potential targets for aminoglycoside binding. As there are variations in the size and position of internal loops and bulges in pre-miRNA, aminoglycosides may bind to different miRNAs with varying preferences. With a view to explore the selective binding potential of aminoglycosides toward a particular pre-miRNA and its subsequent interference of Dicer processing, we have screened 14 FDA-approved, commercially available aminoglycosides, as their pharmacodynamic and pharmacokinetic properties are well understood. We also used two non-aminoglycoside small molecules that have been previously used as antibiotics (amipicillin and erythromycin). Between the two molecules, it is known that erythromycin can bind to RNA, whereas ampicillin cannot. These molecules were screened at a concentration of 5 μM. The change in fluorescence intensity (at 521 nm) in the presence of aminoglycosides was determined by the following equation:

$$\Delta F = (F_{wl} - F_{wol})/F_{wol}$$

where F_{wl} signifies fluorescence intensity in the presence of ligand (aminoglycoside) and F_{wol} signifies intensity in absence of ligand (aminoglycoside). Positive ΔF signifies that the ligand enhances the processing of pre-miRNA, whereas negative ΔF indicates the inhibitory potential of the ligand in pre-miRNA processing.

While ampicillin and erythromycin did not show any significant ΔF, five aminoglycosides (neomycin, amikacin, streptomycin, kanamycin, and tobramycin) showed significant $-\Delta F$ values, indicating their potential to downregulate miR-27a by affecting Dicer processing (Figure 2).

To rule out false positives and false negatives, we performed control experiments. To check whether the inhibitors interact with the beacon, hindering its opening and causing reduced fluorescence, we incubated pre-miR-27a with Dicer for 4 h, as we have already noticed saturation in the level of mature miRNA. This was then added to a mixture of molecular beacon and aminoglycoside, which was pre-incubated for 30 min. Fluorescence intensity was recorded and compared with the no aminoglycoside control (Supplementary Figure S1). We did not find any significant ΔF, which indicates that inhibitory aminoglycosides do not interfere with the molecular beacon.

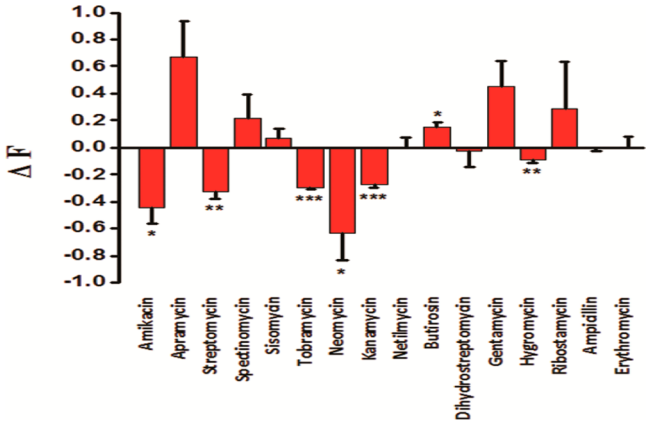

Figure 2. Beacon-based small molecule screening. The beacon and pre-miR-27a were incubated in the absence or presence of small molecules (at a final concentration 5 μM), followed by the addition of Dicer. After 4 h of incubation at room temperate, fluorescence emission spectra were recorded. Fluorescence change (ΔF_{521}) in the case of all small molecules was calculated compared to that with no aminoglycoside. A negative value in ΔF depicts an inhibitory effect of that aminoglycoside in miR-27a maturation, while a positive value indicates an enhancing effect. Error bars represent ±SD, calculated from three independent experiments. ***, $p < 0.0001$; **, $p < 0.0005$; *, $p < 0.005$ (Student's t test).

We then considered if the aminoglycosides somehow stabilize or destabilize miRNA–beacon interaction, thereby causing change in fluorescence. To this end, we used 5 aminoglycosides that showed inhibitory effect (amikacin, neomycin, tobramycin, streptomycin, and kanamycin) and 3 aminoglycosides that showed increased fluorescence (apramycin, gentamycin, and ribostamycin). We incubated pre-miRNA, Dicer, and the molecular beacon for 4 h and observed the fluorescence intensity. Then we added aminoglycoside, observed the fluorescence intensity, and compared them (Supplementary Figure S2). We did not find any significant change in fluorescence upon addition of the aminoglycosides. This indicates that aminoglycosides do not influence fluorescence levels by stabilizing or destabilizing the miR-27a–beacon interaction.

Validation of DNA Beacon-Based Screen. To validate the 'hits' obtained from the screen, we first chose to monitor the perturbation of dicing in an *in vitro* Dicer-blocking assay. The pre-miR-27a was heat-cooled first and then incubated in the presence and absence of the aminoglycosides (final concentration of 5 μM) for 20 min, followed by the addition of Dicer. The reaction mixture was incubated at 37 °C for 90 min and then loaded in the gel to check the miRNA maturation level. If any of the aminoglycosides binds to pre-miR-27a and thereby hinder Dicer processing, the amount of mature miR-27a production would be less as compared to the no aminoglycoside control.

All of the five aminoglycosides, which were positive in the molecular-beacon-based screening, showed differential inhibitory effects (Figure 3). The most potent inhibitors were neomycin and amikacin, which reduced the miR-27a maturation by 25% and 30%, respectively. The other hits from the screen, tobramycin, streptomycin, and kanamycin, showed ~10–20% reduction in miRNA maturation. The varied inhibitory potential could be due to the subtle structural

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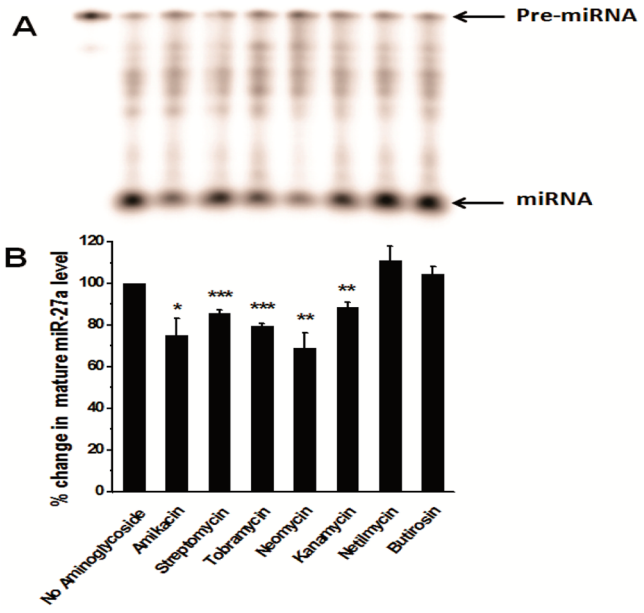

Figure 3. Dicer blocking assay. (A) pre-miR-27a (lane 1), pre-miR-27a with Dicer (lane 2), and pre-miR-27a with Dicer in the presence of different aminoglycosides (lanes 3−9) at a final concentration of 5 μM. (B) Densitometric analysis of miRNA level in the above-mentioned order shows differential effect of these aminoglycosides in Dicer processing and hence miR-27a maturation. Amikacin, streptomycin, tobramycin, neomycin, and kanamycin indeed showed downregulation in mature miR-27a level, while netilmycin and butirosin did not show any effect. Error bars represent ±SD, calculated from three independent experiments. ***, $p < 0.0001$; **, $p < 0.002$; *, $p < 0.005$ (Student's t test).

differences between these aminoglycosides, which could affect their binding affinities to pre-miRNA-27a. We also examined two aminoglycosides (netilmycin and butirosin), which did not show any potential inhibition in the beacon screen, and found no inhibitory effect when assayed for Dicer blockage/hindrance (Figure 3).

Luciferase Screening of Small Molecules. We had previously reported a cell-culture-based luciferase screen for identifying inhibitors of miR-21.[24] Other groups have also previously used *in cellulo* luciferase screening system to identify small molecule inhibitors against miR-21 and miR-122.[20,21] To compare the efficiency of the present *in vitro* DNA-beacon-based screen, we performed a parallel screen using the cell-based assay. The 14 aminoglycosides were screened in the MCF-7 cell line, where the endogenous miR-27a level is high.[27] Cells were transfected with the psiCHECK-2-prohibitin plasmid,[15] followed by aminoglycoside treatment. The construct is designed to express renilla luciferase reporter with the 3′ UTR of prohibitin, a known target of miR-27a.[28] Decrease in the levels of miR-27a, in the presence of an aminoglycoside, would correspond to higher signal intensity of renilla luciferase and *vice versa*.

We performed the luciferase screening at 5 μM concentration of aminoglycosides but did not find any potential inhibitor. It is well reported that the aminoglycosides exhibit poor uptake by mammalian cell lines[37] and could thus lead to lower effective concentration of aminoglycosides inside the cell. At an increased concentration of 20 μM, among the 14 aminoglycosides screened in the dual luciferase assay, amikacin, streptomycin, tobramycin, and neomycin inhibited miR-27a activity (Figure 4). The level of inhibition was comparable to that of an antisense oligonucleotide against miR-27a having five

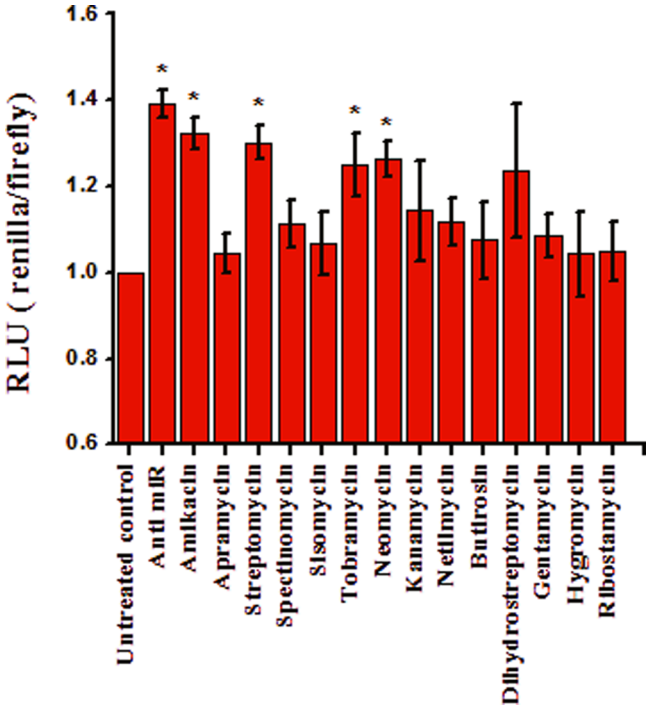

Figure 4. Dual luciferase assay: effect of different aminoglycosides on luciferase signal at a final concentration of 20 μM. Anti-miR-27a (with 5 LNA modifications) at a concentration of 100 nM was used as a positive control. The target 3′ UTR of miR-27a, prohibitin, was downstream of renilla luciferase. All of the renilla luciferase data were normalized with firefly intensity. The experiment was done in the MCF-7 cell line where the endogenous expression of miR-27a is high. Error bars represent ±SD, calculated from three independent experiments. *, $p < 0.0001$ (Student's t test).

LNA modifications (anti-L5miR-27a, 100nM). Except for kanamycin (the least potent inhibitor in the in vitro screen), all of the potential inhibitors from the beacon screen showed inhibitory effects in the cell-based system also.

We further wanted to see whether reconstitution of mature miR-27a in the presence of these four aminoglycosides restores luciferase repression. We overexpressed mature miR-27a in the MCF-7 cell line (using miR-27a mimic) and found reduction in luciferase intensity as compared to the wild type cells. Anti-miR-27a (100 nM) was able to restore the luciferase intensity, while the aminoglycosides (at a concentration of 20 μM) failed to do so (Supplementary Figure S3). This suggests that small molecules perturb the Dicer activity on pre-miRNA forms.

miR-27a Inhibition and Upregulation of Its Target Protein, Prohibitin *in cellulo*. The potential inhibitors were further scrutinized for their miR-27a inhibitory potential inside the cell. To this end, we performed quantitative real time PCR (q-RT-PCR) for mature miR-27a levels upon treatment with these compounds in MCF-7 cells where miR-27a is overexpressed.[27] MCF-7 cells were treated with 20 μM concentration of the five potential inhibitors (amikacin, neomycin, kanamycin, tobramycin, and streptomycin) and two other aminoglycosides (netilmycin and butirosin) that did not show any effect in beacon-based *in vitro* screening. After 48 h of incubation, cellular RNA was isolated, and q-RT-PCR was performed to ascertain the extent of downregulation of miR-27a. Neomycin,

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tobramycin, streptomycin, and amikacin indeed showed ~35−50% downregulation of mature miR-27a level compared to untreated control, while kanamycin, butirosin, and netilmycin did not show any significant effect (Figure 5). Streptomycin

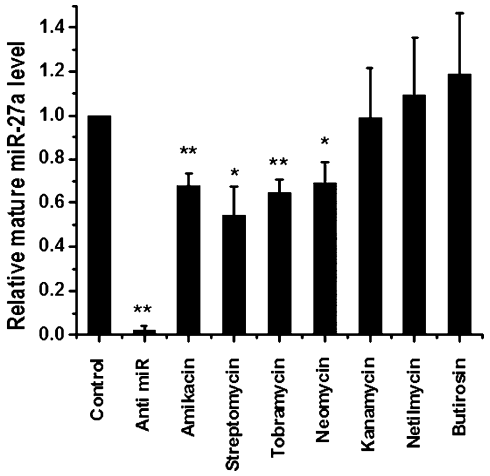

Figure 5. Relative level of mature miR-27a, determined by qRT-PCR. Relative levels of mature miR-27a shown in the presence different aminoglycosides (final concentration of 20 μM). Five LNA modified anti-miR-27a (100 nM) was taken as a positive control. Amikacin, streptomycin, tobramycin, and neomycin showed significant decrease in mature miR-27a level, while kanamycin, netilmycin, and butirosin did not show any significant effect. Error bars represent ±SD, calculated from three independent experiments. **, $p < 0.0005$; *, $p < 0.002$ (Student's t test).

shows comparatively effective downregulation of miR-27a, which corroborates our previous observations (data not shown) on miR-21.[24] Our data suggest that the beacon screen is sufficient to identify potential inhibitors of miR-27a function by downregulation of mature miRNA levels, reiterating the accuracy of the screen.

The four aminoglycosides that inhibit miR-27a processing *in cellulo* exhibit ~30−50% downregulation compared to anti-miR-27a (>95%), but anti-miR-27a and the inhibitory aminoglycosides have comparable effects in the luciferase assay. To check the off-target effect we looked for miRNAs that are predicted to target 3′ UTR of PHB. We used Pictar, Targetscan, and Miranda and took a list of consensus miRNAs. Apart from miR-27a, three other miRNAs, namely, miR-27b, miR-205, and miR-128, were predicted to target PHB 3′ UTR. We checked the expression of miR-27b, miR-205, and miR-128 in aminoglycoside-treated samples and found that there was no significant change in their levels in amikacin-, streptomycin-, tobramycin-, and neomycin-treated samples, compared to untreated control (Supplementary Figure S4). So we concluded that other miRNAs targeting PHB does not have any role in modulating luciferase activity as they were unaffected upon aminoglycoside treatment.

We also checked the endogenous level of prohibitin (target of miR-27a) in the presence of the potential inhibitors. At a concentration of 20 μM all four potential aminoglycoside inhibitors showed significant upregulation of prohibitin compared to the untreated control (Figure 6). Anti-miR-27a (100 nM) was taken as a positive control.

Identification of the Binding Region of the Aminoglycosides to pre-miR-27a. Given that these aminoglycosides affect miRNA maturation by hindering Dicer processing,

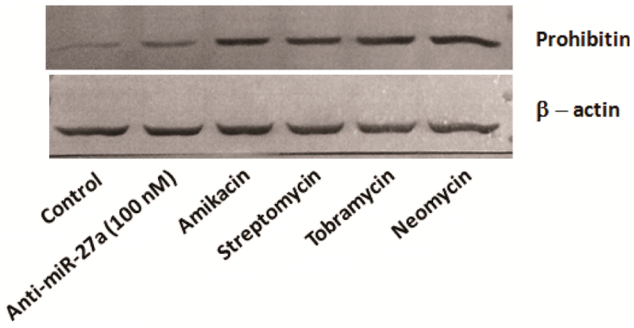

Figure 6. Western blot detection of prohibitin (PHB) shows increased levels upon amikacin, streptomycin, tobramycin, and neomycin treatment (20 μM). Anti-miR-27a (100 nM) was taken as positive control.

we sought to determine the binding interactions of these aminoglycosides to pre-miR-27a. We used neomycin and streptomycin as representatives of the potential inhibitors owing to their consistent efficacy in various confirmatory assays and did enzymatic footprinting in the presence of increasing concentration of neomycin and streptomycin (0−15 μM). S1 nuclease enzymatic probing was performed to identify the binding regions of the aminoglycosides. Briefly, S1 nuclease cleaves single-stranded nucleic acids endonucleolytically to produce 5′-phosphoryl-terminated end products. We noted that in the presence of streptomycin and neomycin the cleavage of pre-miR-27a by S1 nuclease was reduced. S1 nuclease probing indicated the binding of neomycin and streptomycin to the stem loop junction (protected residues G29 to U55 are highlighted in red and numbered in green in Figures 7 and 8). These results indicated binding of neomycin and streptomycin to pre-miR-27a at regions close to the terminal loop. Such binding may obstruct Dicer binding and processing of the pre-miR27a into its mature form. Collectively, these observations confirm the results obtained by the *in vitro* beacon-based screening for miR-27a inhibitors of miRNA expression and function.

Conclusion. Aberrant expression of miRNA is well reported in different diseases and pathological conditions. About 40% of aberrantly expressed miRNAs in cancer show overexpression. So ligands specific to pre-miRNA that can block Dicer processing can be of great therapeutic importance. The need for alternate methods for miRNA intervention has paved the way for development of several platforms for screening small molecule inhibitors of miRNA expression and function.

Here, we report a novel fluorescent molecular-beacon-based *in vitro* high-throughput screening assay to identify scaffolds that downregulate miRNA levels by hindering Dicer processing. All of the materials that are needed to set up the screening assay, such as the beacon, Dicer enzyme, and pre-miRNA, are commercially available, and the screening assay can be easily set up in any laboratory. Our assay uses a DNA-based beacon that is specific to the mature miRNA alone, making it amenable to test for any number of compounds that might inhibit the miRNA maturation. The high-throughput beacon-based assay is cost-effective, sensitive, and robust and compares favorably to a previously described *in vitro* high-throughput method[19] in its ability to identify small molecule modulators of Dicer activity. In a pilot screen of 14 aminoglycosides that have RNA-binding properties, we identified five potential inhibitors of miR-27a processing. These inhibitory molecules were further examined

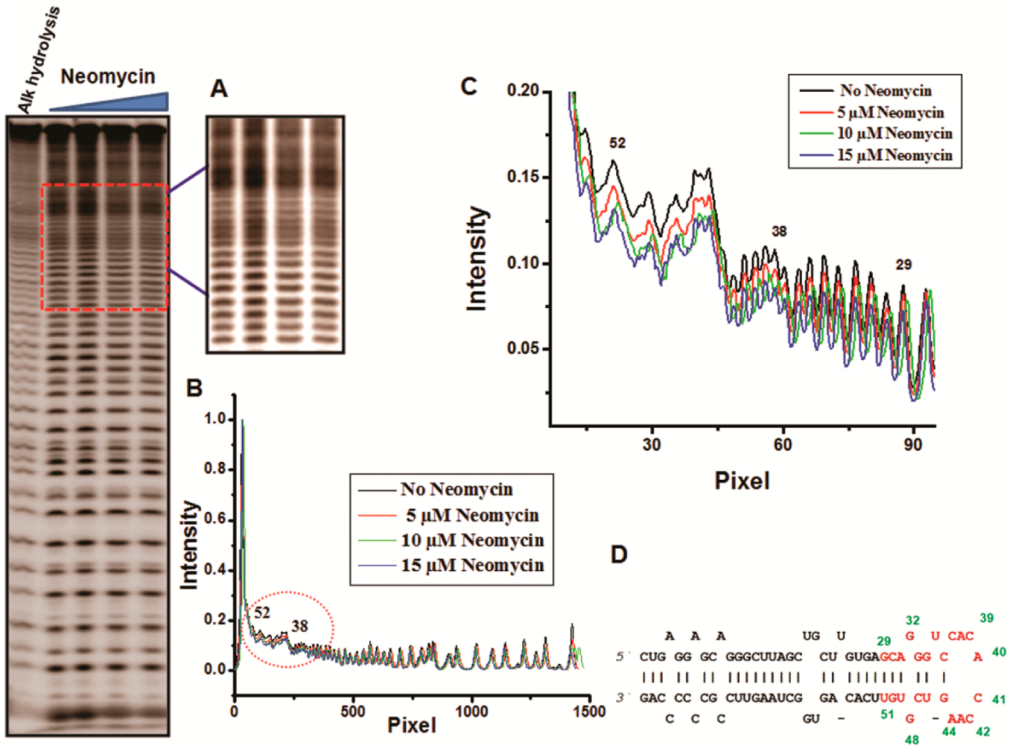

Figure 7. S1 nuclease footprinting: S1 nuclease probing of pre-miR-27a and neomycin interaction. (A) Lane 1 from the left shows alkaline hydrolysis ladder. Cleavage pattern of pre-miR-27a (lane2) and pre-miR-27a complexed with 5 μM, 10 μM, and 15 μM neomycin (lanes 3−5). Enlargement indicates the portion of the gel where there was a protection in the presence of neomycin. (B) S1 nuclease cleavage pattern of uncomplexed pre-miR-27a (lane2) and pre-miR-27a complexed with 5 μM, 10 μM, and 15 μM neomycin (lanes 3−5). (C) Representative graph plot of the intensity of bands from nucleotides 28−55 for uncomplexed pre-miR-27a (black line) and pre-miR-27a complexed with 5 μM, 10 μM, and 15 μM neomycin (red, green, and blue lines, respectively). (D) The sites of protection were mapped to pre-miR-27a secondary structure (colored in red and residues indicated in green).

to confirm their Dicer-blocking properties. Of the five initially identified, streptomycin, neomycin, and tobramycin proved to be equally effective in a cell-based luciferase assay that has been described earlier. The identified molecules were also able to effectively downregulate miR27a *in cellulo* as gauged by qRT-PCR. Subsequent determination of binding sites of two representative inhibitors (streptomycin and neomycin) provides a basis for their observed effects on miR27a. Thus, small molecule ligands identified by this screening assay not only show their inhibitory effect *in vitro* but also shed light on the most probable mechanism of inhibition *in cellulo*, paving the way for improvement of the identified small molecules to their full therapeutic potential.

METHODS

Molecular-Beacon-Based Small Molecule Screening. HPLC purified anti-miR beacon was purchased from Sigma. The sequences of the beacon: 5′xCGCGCGAGCGGAACTTAGCCACTGTGAAT-CGCGCGy3 (x = FAM, fluorophore, y = BHQ-1, quencher). For the molecular beacon study the final concentrations of anti-miR beacon and pre-miR-27a were 50 nM and 25 nM, respectively, in a reaction volume of 100 μL. The reaction buffer contains 10 mM sodium cacodylate, 1 mM $MgCl_2$, and 10 mM NaCl (pH 7.5). Dicer (Genlantis) was added at a concentration of 0.5 U. Initially the beacon in the presence of pre-miR-27a and beacon with Dicer were incubated for 30 min, and the fluorescence spectra were measured in a Fluorolog 3 (Horiba) spectrofluorophotometer equipped with a thermoelectrically temperature-controlled cell holder (quartz cuvette, 1 cm × 1 cm). The excitation wavelength was set at 492 nm, and the emission spectra were recorded from 510 to 590 nm (as the excitation wavelength of FAM is 492 nm and emission maxima is at 521 nm). The excitation and emission slit widths were 2 and 5 nm, respectively. For the time-dependent Dicer kinetics, pre-miR-27a with molecular beacon was incubated for 10 min, followed by the addition of Dicer (Genlantis). Fluorescence spectra were taken at regular time intervals, starting from 5 min till 16 h.

For beacon-based small molecule screening, beacon and pre-miR-27a was first incubated, followed by addition of aminoglycosides (Sigma) at a final concentration of 5 μM. This mixture was was incubated for 30 min at RT before addition of Dicer, and the fluorescence spectra were collected after 4 h. The change in fluorescence intensity (at 521 nm) in the presence of aminoglycosides was determined by the following equation:

$$\Delta F = (F_{wl} - F_{wol})/F_{wol}$$

where F_{wl} signifies fluorescence intensity in the presence of ligand (aminoglycoside), and F_{wol} signifies intensity in absence of ligand (aminoglycoside).

Dicer Blocking Assay. P^{32}-5′ end-labeled RNA (40000 cpm, ∼10 ng) was incubated in 10 mM sodium cacodylate buffer, 1 mM $MgCl_2$, and 10 mM NaCl (pH 7.5) in a total volume of 8 μL. The reaction mixture was heated 90 °C for 5 min followed by slow cooling to 37 °C. One microliter of aminoglycoside was added to a final concentration of 5 μM and incubated for 20 min, followed by addition of 1 μL (0.03 units) of turbo Dicer (Genlantis). The assay was performed at 37 °C for 90 min. Reaction was stopped by adding Dicer stop buffer (Genlantis). Ten microliters of 95% formamide with dyes (Xylene Cyanol and Bromophenol Blue) was added and run in a 15% denaturing PAGE. The gel was exposed to phosphor screen overnight, and the image was taken using Typhoon trio phosphoimager.

Luciferase Reporter Assay. The *in cellulo* dual luciferase screening was done using psiCHECK-2-prohibitin vector, as described

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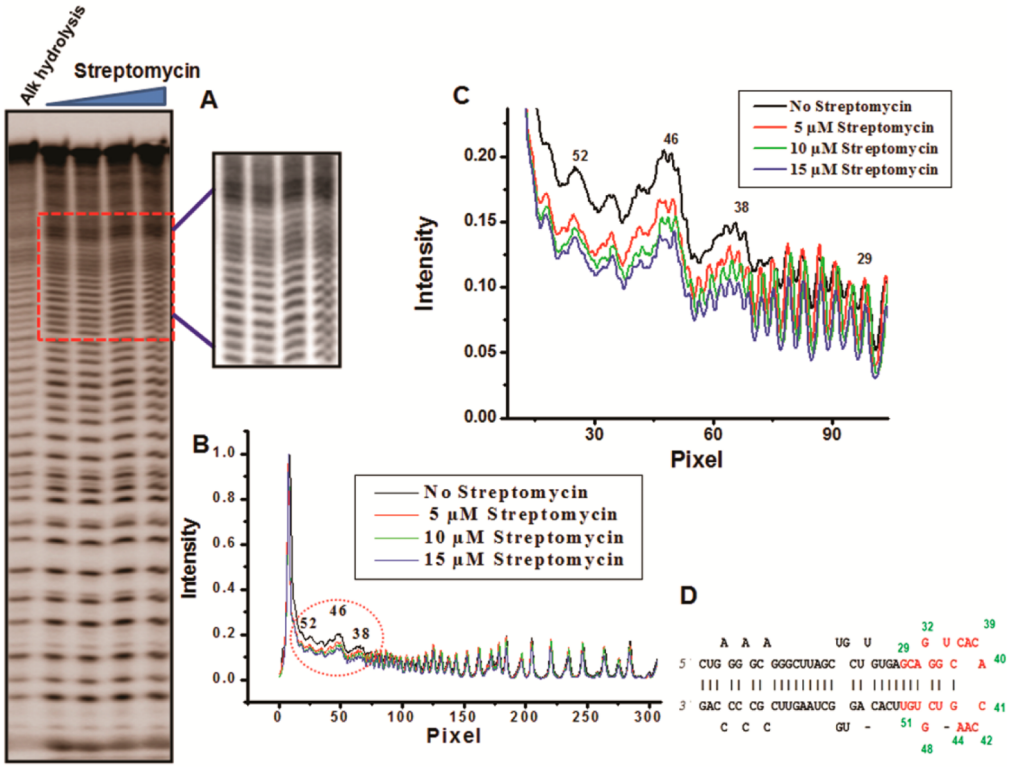

Figure 8. S1 nuclease footprinting: S1 nuclease probing of pre-miR-27a and streptomycin interaction. (A) Lane 1 from the left shows alkaline hydrolysis ladder. Cleavage pattern of pre-miR-27a (lane2) and pre-miR-27a complexed with 5 μM, 10 μM, and 15 μM streptomycin (lanes 3−5). Enlargement indicates the portion of the gel where there was a protection in the presence of strepotomycin. (B) S1 nuclease cleavage pattern of uncomplexed pre-miR-27a (lane2) and pre-miR-27a complexed with 5 μM, 10 μM, and 15 μM streptomycin (lanes 3−5). (C) Representative graph plot of the intensity of bands from nucleotides 28−55 for uncomplexed pre-miR-27a (black line) and pre-miR-27a complexed with 5 μM, 10 μM, and 15 μM streptomycin (red, green, and blue lines, respectively). (D) The sites of protection were mapped to pre-miR-27a secondary structure (colored in red and residues indicated in green).

previously,[15] where the 3′ UTR of prohibitin (target of miR-27a) is cloned downstream of renilla luciferase and firefly luciferase is for normalization. The presence of miR-27a will cause decrease in the renilla luciferase signal. The MCF-7 cell line was the cell line of choice as miR-27a is reported to be overexpressed in MCF-7. Cells were cultured in DMEM high-glucose medium (Gibco) containing 10% FBS (without antibiotics and antimycotics) at 37 °C in a humidified incubator with 5% CO_2. Approximately 4×10^4 cells were seeded in each well of a 24-well plate, 24 h prior to transient transfection. Transfection was done at around 75% confluency. The pSilencer-miR-27a vector was transfected using Lipofectamine 2000 transfection reagent (Invitrogen). Anti-miR-27a and miR-27a mimic (Qiagen), at a final concentration of 100 nM, were also transfected using Lipofectamine 2000. All the transfections are done in triplicate and repeated twice for statistical analysis. The cells were incubated at 37 °C for 4 h followed by the replacement of transfection media with standard growth media (500 μL), supplemented with 20 μM aminoglycosides. At 48 h after treatment, cells were lysed in PLB buffer (Promega). The luciferase assay was performed using the dual-luciferase reporter assay kit (Promega). Renilla luciferase values were normalized using firefly luciferase values.

***In Vitro* Transcription of pre-miR-27a.** The pre-miR-27a RNA was made from template oligonucleotides (Sigma) using Megascript High yield transcription kit (Ambion). To synthesize the duplex template, forward primer 5′TAATACGACTCACTATAGGGCTGAGGAGCAGGGCTTAGCTGCTTGTGAGCAGGGTCCACACCAAGTCGTGTTCACAGTGG 3′ and reverse primer 5′CTGGGGGGCGGAACTTAGCCACTGTGAACACGACTTGGTGTGGACCCTGCTCACAAGCAGCTAAGCCCTGCTCCTCAGCC 3′ were subjected to primer extension. Each of the primers at 2 μM concentration were added to a reaction mixture containing Taq polymerase (5 U), dNTPs (0.2 mM), Taq polymerase buffer (1X), and $MgCl_2$ (2 mM). The reaction mixture was heated at 95 °C for 5 min to denature, followed by snap-chilling on ice for 10 min, followed by primer extension incubation at 72 °C for 30 min. The hybrid template with T7 promoter (sequence highlighted in blue) was first gel checked for its proper size and then was used for *in vitro* transcription following manufacturer's instructions (Ambion, Inc.). The pre-miR-27a substrate was gel purified using 15% denaturing PAGE.

Quantitative Real Time PCR. MCF-7 cells maintained in growth medium (DMEM high-glucose, 10% FBS, without antibiotic and antimycotic) were seeded in 24 well plate (4×10^4 cells/well) and at a confluency of ∼70% treated with aminoglycosides (Sigma) at a final concentration of 20 μM. Cells were incubated at 37 °C humidified incubator with 5% CO_2 for 48 h after treatment. The media was removed, cells were washed with PBS, and RNA isolation was done using TRizol Reagent (Invitrogen). Expression of miR-27a was quantified using QuantiMir kit. (SBI, catalog number RA660A-1). Primers used for qRT-PCR were forward primer, TTCACAGTGGCTAAGTTCCGC, and reverse primer, QuantiMir universal reverse primer. Forward primer was purchased from Sigma, and the universal reverse primer was provided in the QuantiMir kit. Sybr-green I PCR master mix (Applied biosystems) was used to quantify mature miR-27a levels on Roche Lightcycler 480 and the data was normalized with respect to the reference gene U6. Data analysis was done by using 2(-Delta Delta C(T)) method.[38]

Western Blot. Total protein was isolated from treated and untreated MCF-7 cells using cell lysis RIPA buffer (Pierce Chemical Co., Rockland, IL). Five LNA modified anti-miR-27a (100 nM) transfected sample was taken as positive control. Protein concentration was estimated using BCA protein assay (Pierce Chemical Co., Rockland, IL). An equivalent amount of protein from each sample was resolved on 12% SDS-PAGE. Following transfer to nitrocellulose membrane and blocking with 5% nonfat milk, the blot was incubated

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with primary antibody (1:500) specific for Prohibitin (PHB) protein (Abcam). After washing the blot with 1X TBS, it was probed with alkaline phosphatase Conjugated secondary antibody (1:2000 dilution) (Abcam). The blot was developed using AP developing solution (B genie).

Enzymatic Footprinting. P^{32}-5′ end labeled pre-miR-27a was subjected to a denaturation step before structure probing. The P^{32}-5′ end-labeled RNA (60000 cpm) was incubated in 10 mM sodium cacodylate buffer, 1 mM $MgCl_2$, and 10 mM NaCl (pH 7.5) in a total volume of 8 μL. The sample was heated to 95 °C for 5 min and allowed to slow cool to 37 °C. One microliter of aminoglycosides was add to a final concentration of 5 μM, 10 μM, and 15 μM and incubated for 20 min. One microliter of S1 nuclease (47.5 U) (Fermentas) was added and incubated at 37 °C for 90 s. Reaction was stopped by adding 95% formamide with dyes followed by immediate snap -chilling on dry ice. An alkaline digestion ladder was generated as described by Regulski et al.[39] Samples were run in 15% denaturing PAGE and exposed to phosphor screen overnight. Images were obtained using typhoon trio phosphoimager. Cleavage profiles were generated using ImageQuaNT software.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

This material is available free of charge *via* the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: souvik@igib.res.in.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Bartel, D. P. (2009) MicroRNAs: target recognition and regulatory functions. *Cell 136*, 215−233.

(2) Kim, V. N., Han, J., and Siomi, M. C. (2009) Biogenesis of small RNAs in animals. *Nat. Rev. Mol. Cell. Biol. 10*, 126−139.

(3) Abbott, A. L. (2011) Uncovering new functions for microRNAs in Caenorhabditis elegans. *Curr. Biol. 21*, R668−R671.

(4) Mendell, J. T., and Olson, E. N. (2012) MicroRNAs in stress signaling and human disease. *Cell 148*, 1172−1187.

(5) Contreras, J., and Rao, D. S. (2012) MicroRNAs in inflammation and immune responses. *Leukemia 26*, 404−413.

(6) Tiscornia, G., and Izpisua Belmonte, J. C. (2010) MicroRNAs in embryonic stem cell function and fate. *Genes Dev. 24*, 2732−2741.

(7) Rottiers, V., and Naar, A. M. (2012) MicroRNAs in metabolism and metabolic disorders. *Nat. Rev. Mol. Cell. Biol. 13*, 239−250.

(8) Zampetaki, A., and Mayr, M. (2012) MicroRNAs in vascular and metabolic disease. *Circ. Res. 110*, 508−522.

(9) Esteller, M. (2011) Non-coding RNAs in human disease. *Nat. Rev. Genet. 12*, 861−874.

(10) Medina, P. P., Nolde, M., and Slack, F. J. (2010) OncomiR addiction in an in vivo model of microRNA-21-induced pre-B-cell lymphoma. *Nature 467*, 86−90.

(11) Boutla, A., Delidakis, C., and Tabler, M. (2003) Developmental defects by antisense-mediated inactivation of micro-RNAs 2 and 13 in Drosophila and the identification of putative target genes. *Nucleic Acids Res. 31*, 4973−4980.

(12) Krutzfeldt, J., Rajewsky, N., Braich, R., Rajeev, K. G., Tuschl, T., Manoharan, M., and Stoffel, M. (2005) Silencing of microRNAs in vivo with 'antagomirs'. *Nature 438*, 685−689.

(13) Ebert, M. S., Neilson, J. R., and Sharp, P. A. (2007) MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. *Nat. Methods 4*, 721−726.

(14) Jadhav, V. M., Scaria, V., and Maiti, S. (2009) Antagomirzymes: oligonucleotide enzymes that specifically silence microRNA function. *Angew. Chem. 48*, 2557−2560.

(15) Kaur, H., Scaria, V., and Maiti, S. (2010) ″Locked onto the target″: increasing the efficiency of antagomirzymes using locked nucleic acid modifications. *Biochemistry 49*, 9449−9456.

(16) Castanotto, D., and Rossi, J. J. (2009) The promises and pitfalls of RNA-interference-based therapeutics. *Nature 457*, 426−433.

(17) Guan, L., and Disney, M. D. (2012) Recent advances in developing small molecules targeting RNA. *ACS Chem. Biol. 7*, 73−86.

(18) Thomas, J. R., and Hergenrother, P. J. (2008) Targeting RNA with small molecules. *Chem. Rev. 108*, 1171−1224.

(19) Davies, B. P., and Arenz, C. (2006) A homogenous assay for micro RNA maturation. *Angew. Chem. 45*, 5550−5552.

(20) Gumireddy, K., Young, D. D., Xiong, X., Hogenesch, J. B., Huang, Q., and Deiters, A. (2008) Small-molecule inhibitors of microrna miR-21 function. *Angew. Chem. 47*, 7482−7484.

(21) Young, D. D., Connelly, C. M., Grohmann, C., and Deiters, A. (2010) Small molecule modifiers of microRNA miR-122 function for the treatment of hepatitis C virus infection and hepatocellular carcinoma. *J. Am. Chem. Soc. 32*, 7976−7981.

(22) Deiters, A. (2010) Small molecule modifiers of the microRNA and RNA interference pathway. *AAPS J. 12*, 51−60.

(23) Georgianna, W. E., and Young, D. D. (2011) Development and utilization of non-coding RNA-small molecule interactions. *Org. Biomol. Chem. 9*, 7969−7978.

(24) Bose, D., Jayaraj, G., Suryawanshi, H., Agarwala, P., Pore, S. K., Banerjee, R., and Maiti, S. (2012) The tuberculosis drug streptomycin as a potential cancer therapeutic: inhibition of miR-21 function by directly targeting its precursor. *Angew. Chem. 51*, 1019−1023.

(25) Tan, G. S., Chiu, C. H., Garchow, B. G., Metzler, D., Diamond, S. L., and Kiriakidou, M. (2012) Small molecule inhibition of RISC loading. *ACS Chem. Biol. 7*, 403−410.

(26) Mertens-Talcott, S. U., Chintharlapalli, S., Li, X., and Safe, S. (2007) The oncogenic microRNA-27a targets genes that regulate specificity protein transcription factors and the G2-M checkpoint in MDA-MB-231 breast cancer cells. *Cancer Res. 67*, 11001−11011.

(27) Guttilla, I. K., and White, B. A. (2009) Coordinate regulation of FOXO1 by miR-27a, miR-96, and miR-182 in breast cancer cells. *J. Biol. Chem. 284*, 23204−23216.

(28) Liu, T., Tang, H., Lang, Y., Liu, M., and Li, X. (2009) MicroRNA-27a functions as an oncogene in gastric adenocarcinoma by targeting prohibitin. *Cancer Lett. 273*, 233−242.

(29) Chintharlapalli, S., Papineni, S., Abdelrahim, M., Abudayyeh, A., Jutooru, I., Chadalapaka, G., Wu, F., Mertens-Talcott, S., Vanderlaag, K., Cho, S. D., Smith, R., III., and Safe, S. (2009) Oncogenic microRNA-27a is a target for anticancer agent methyl 2-cyano-3,11-dioxo-18beta-olean-1,12-dien-30-oate in colon cancer cells. *Int. J. Cancer 125*, 1965−1974.

(30) Persat, A., and Santiago, J. G. (2011) MicroRNA profiling by simultaneous selective isotachophoresis and hybridization with molecular beacons. *Anal. Chem. 83*, 2310−2316.

(31) Baker, M. B., Bao, G., and Searles, C. D. (2012) In vitro quantification of specific microRNA using molecular beacons. *Nucleic Acids Res. 40*, e13.

(32) Flores-Jasso, C. F., Arenas-Huertero, C., Reyes, J. L., Contreras-Cubas, C., Covarrubias, A., and Vaca, L. (2009) First step in pre-miRNAs processing by human Dicer. *Acta Pharmacol. Sin. 30*, 1177−1185.

(33) MacRae, I. J., Zhou, K., and Doudna, J. A. (2007) Structural determinants of RNA recognition and cleavage by Dicer. *Nat. Struct. Mol. Biol. 14*, 934−940.

(34) MacRae, I. J., Zhou, K., Li, F., Repic, A., Brooks, A. N., Cande, W. Z., Adams, P. D., and Doudna, J. A. (2006) Structural basis for double-stranded RNA processing by Dicer. *Science 311*, 195−198.

(35) Park, J. E., Heo, I., Tian, Y., Simanshu, D. K., Chang, H., Jee, D., Patel, D. J., and Kim, V. N. (2011) Dicer recognizes the 5′ end of RNA for efficient and accurate processing. *Nature 475*, 201−205.
(36) Wang, Y., and Rando, R. R. (1995) Specific binding of aminoglycoside antibiotics to RNA. *Chem. Biol. 2* (5), 281−290.
(37) Luedtke, N. W., Carmichael, P., and Tor, Y. (2003) Cellular uptake of aminoglycosides, guanidinoglycosides, and poly-arginine. *J. Am. Chem. Soc. 125*, 12374−12375.
(38) Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods 25*, 402−408.
(39) Regulski, E. E., and Breaker, R. R. (2008) In-line probing analysis of riboswitches. *Methods Mol. Biol. 419*, 53−67.