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The Tuberculosis Drug Streptomycin as a Potential Cancer Therapeutic: Inhibition of miR-21 Function by Directly Targeting Its Precursor**

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Dedicated to Professor Samir K. Brahmachari on the occasion of his 60th birthday

MicroRNAs (miRNAs) play crucial roles in regulating gene expression in many cellular contexts. Deregulation of miRNAs has been implicated in a number of disease conditions and thus, small molecules that can modulate mature miRNA levels in cells can have immense therapeutic potential. Aminoglycosides, mostly used as antibiotics, are known specifically to bind to certain RNA secondary structures. Herein, we report that one such aminoglycoside, streptomycin, can down-regulate the levels of mature miR-21, a miRNA with roles in a variety of cancers. We suggest that streptomycin down-regulates miR-21 by binding to pre-miRNA (its precursor) and blocking the function of the Dicer enzyme, an essential step in miRNA maturation.

MicroRNAs are a class of endogenous noncoding RNAs that act post-transcriptionally to target mRNAs for translational repression, cleavage, and destabilization.[1] A single miRNA has been predicted to regulate hundreds of mRNAs; therefore miRNAs may control as much as 60% of all human genes.[2] miRNAs have been shown to play important regulatory roles in spatio-temporal patterning of the vertebrate body, as well as adult physiology. Aberrant expression of miRNAs is well documented in several pathological states, most notably in the context of cancer. About 40% of aberrantly expressed miRNAs in cancer show overexpression,[3] thus making them a novel class of therapeutic targets. Therefore, methods for altering the levels of miRNA expression are one of the focuses of drug development.[4] Specific knockdown of the target miRNAs by anti-miRNA oligonucleotides (antagomirs) have been explored both in vitro and in vivo to this end.[5] However, effective delivery of such agents into target tissues remain a major hurdle for nucleic-acid-based therapies.[6] Thus the development of alternate and nonconventional methods for modulating specific miRNA levels would be of great value. Recently, Gumireddy et al.[7] and Young et al. reported the first use of small molecules as inhibitors of miRNA, specifically miR-21 and miR-122, function.[8] Independently, the group of Arenz has shown that kanamycin,[9,10] 2-deoxy-streptamine (2-DOS), and neamine[11] can block let-7 processing by binding to pre-miR and thus block Dicer activity. Lunse et al. reported an aptamer that inhibits pri-miRNA processing by targeting the apical loop domain of pri-miRNA.[12] Others[13,14] have also provided evidence that the small molecule enoxacin modulates miRNA processing by enhancing TRBP (TAR RNA binding protein) activity in Dicer processing. Although, these studies provide a proof of concept, additional studies for understanding the modes of action, specificity, toxicity, and pharmacodynamic and pharmacokinetic properties of these molecules are called for.[15]

MicroRNAs are produced in a series of stereotypical steps which start with a long primary transcript from the nucleus and result in an approximately 22nt, mature, and functional miRNA in the cytosol. The long primary transcript (pri-miRNA) which can be several kilobases long is processed into a smaller (ca. 70nts) precursor (pre-miRNA) by the microprocessor complex, which upon export to cytoplasm via Exportin 5-Ran GTP complex becomes a substrate for the Dicer enzyme. Processing by the Dicer complex produces the mature duplex miRNA.[16]

Aminoglycosides, a number of which are used as antibiotics, are known to bind to secondary structures of RNA, particularly at stem-loops and bulges.[17] The miRNA precursors are known to form stem-loops with bulges as they fold into hairpinlike intermediates during their maturation and processing by Dicer.[18] Thus, we explored the potential of aminoglycoside(s) to inhibit miRNA function. We chose the oncogenic miRNA miR-21 to test this hypothesis, as it is known to be overexpressed in breast, lung, colon, and prostate cancers.[19]

We screened fifteen known aminoglycosides (see Table S1 in the Supporting Information) in cultured cells transfected with the pEZX-MT01 plasmid (Genecopoeia Inc). The construct is designed to express firefly luciferase reporter

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with the 3′UTR of PDCD4 (Programmed cell death 4), a known target of miR-21.[20] Decrease in the levels of miR-21 will correspond to higher signal intensity of firefly luciferase and vice versa. The assay (Dual Luciferase Kit, Promega) was performed on the MCF-7 cell line that expresses high levels of endogenous miR-21.[21]

We found that among the aminoglycosides screened, streptomycin was a highly effective inhibitor, inhibiting miR-21 activity at a concentration of 10 μM (see Figure S1 in the Supporting Information). The level of inhibition was comparable to that of an antisense oligonucleotide against miR-21 having four LNA modifications (Anti L4miR-21, 100 nM). To determine the extent of down-regulation of miR-21 expression by streptomycin, quantitative RT-PCR was performed. After treating cells with streptomycin at concentrations ranging from 200 nM to 5 μM (Figure 1) for 48 hours, the total RNA was isolated (TRIzol, Invitrogen) and RT-PCR was carried out using QuantiMir primers (SBI) against

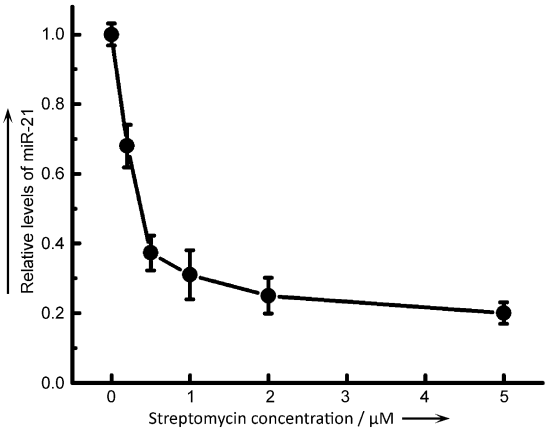

Figure 1. Relative levels of mature miR-21 shown at increasing concentrations of streptomycin treatment (200 nM to 5 μM).

mature miR-21. Indeed a significant decrease in miR-21 levels was observed compared to the untreated control cells, with the lowest miRNA levels occurring at 5 μM streptomycin. To assess the specificity of streptomycin towards miR-21, the levels of nine other oncogenic miRNAs were determined by quantitative RT-PCR in samples treated with 5 μM streptomycin. We observed that miR-210, miR-30a, miR-103, miR-95, miR-221, miR-223, miR-155, and miR-143 remained unchanged, with the exception of miR-27a which was down-regulated (see Figure S2A in the Supporting Information). RT-PCR end products were analyzed on the gel which gave us single bands of appropriate length corresponding to mature miRNAs (see Figure S2B).

Next, we asked whether streptomycin could be used to antagonize miR-21 function. We measured the expression of one of the principle targets of miR-21, the programmed cell death protein 4 (PDCD4), in streptomycin treated and untreated cells (Figure 2A). Densitometry analysis of the western blots revealed a 1.6- to 2-fold (Figure 2B) increase in the levels of the PDCD4 upon treatment with 1 μM, 2 μM, and 5 μM streptomycin in comparison to the untreated controls. A

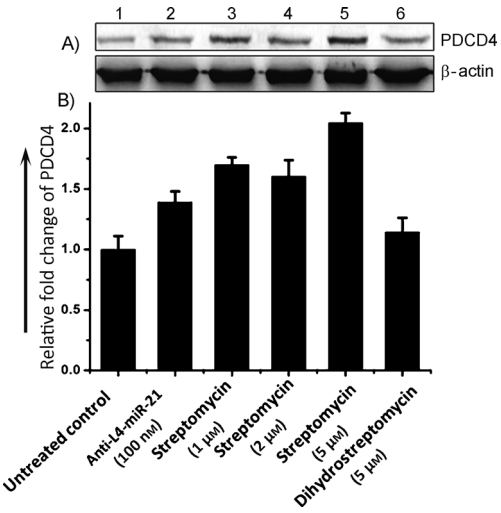

Figure 2. A) Western blot detection of PDCD4 shows increased levels upon streptomycin treatment (lane 1: untreated; lane 2: Anti L4; lanes 3, 4, and 5: streptomycin at 1, 2, and 5 μM, respectively; lane 6: dihydrostreptomycin 5 μM). B) Relative fold-change analysis shows streptomycin was comparatively more effective than Anti L4-21, while control aminoglycoside dihydrostreptomycin had almost no effect.

structurally related aminoglycoside, dihydrostreptomycin did not show any change in PDCD4 level compared to untreated control (Figure 2B). To check the status of the expression level of PDCD4 in a condition where miR-21 is absent or low, we repeated the assay in a Jurkat cell line where its expression is basal.[22,23] We did not find any significant change in PDCD4 levels upon streptomycin and dihydrostreptomycin treatment (see Figure S3 in the Supporting Information). This result implies that the change in PDCD4 level upon streptomycin treatment is a result of miR-21 inhibition.

Contrary to previous reports of small-molecule-mediated inhibition of miRNAs where indirect inhibition is observed,[15] we show that the mode of action of streptomycin-mediated inhibition could be direct, by performing thermal melting experiments (Figure 3) with in vitro transcribed (MEGA-Script T7, Ambion) pre-miR-21. We note that the melting curve of free pre-miR-21 shows two melting domains with differential thermal stability. We correlated the curve characteristics to the M-Fold prediction (see Figure S4 in the

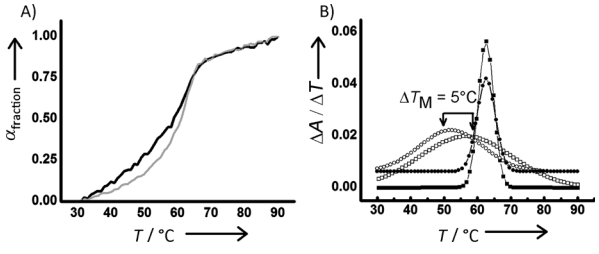

Figure 3. A) UV melting analysis of free (175 nM; black line) and streptomycin-bound miR-21 (1.75 μM; gray line). B) Differential curve analysis showing the first melting domain [free (○), streptomycin bound (□)] and second melting domain [free (●), streptomycin bound (■)]. Differential curve analysis of first melting domain showed stabilization of 5 °C in presence of streptomycin.

Supporting Information) of the secondary structure of pre-miR-21, thus suggesting that the lower (less-stable) melting domain might correspond to the region of the terminal loop. Upon addition of streptomycin, we observed a thermal stabilization (ca. 5 °C) at the lower melting domain as shown in the differential curve analysis of the free and streptomycin-bound pre-miR-21.

We also performed a docking study between pre-miR-21 and all the aminoglycosides used in the screen, and streptomycin showed the strongest binding to pre-miR-21 (see Table S2 in the Supporting Information). We then sought to determine the precise binding site (region) of streptomycin to pre-miR-21. We analyzed the 10 top scoring dock structures and arrived at a consensus streptomycin/pre-miR-21 dock structure that predicted that streptomycin docking with the pre-miR-21 at a region close to the terminal loop (see Figure S5).The streptose ring of streptomycin is capsulated by stacked arrays of bases from both loops and bulges of the pre-miRNA. Furthermore, the streptomycin structure spans the region from residues G28 to G44 and forms eight hydrogen bonds, with an average distance of 2.32 Å, with residues G44, U43, A42, A37, and G28 in the pre-miRNA (see Table S3). Thus, the specificity is borne from the direct hydrogen-bond formation between the streptose functional groups and base edges that line the inner walls of the cylindrical binding pocket; which is in accordance with crystal structure information of streptomycin bound to an RNA aptamer.[24]

Using S1 nuclease (Figure 4A) and RNase T1 cleavage pattern analysis (see Figure S6 in thte Supporting Information) we tested whether streptomycin binds at the predicted regions of the miR-21. S1 nuclease cleaves single-stranded nucleic acids endonucleolytically to produce 5′-phosphoryl-terminated products. The presence of streptomycin (2 μM and 5 μM) reduced the cleavage of the RNA by S1 nuclease (Figure 4B), thus indicating the binding of streptomycin to

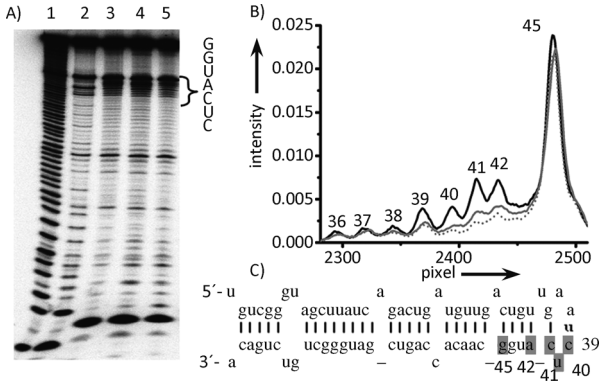

Figure 4. S1 nuclease probing of pre-miR-21 and streptomycin interaction. A) Cleavage pattern of uncomplexed pre-miR-21 (lane 3) and pre-miR-21 complexed with 2 μM and 5 μM streptomycin (lanes 4 and 5, respectively). Lanes 1 and 2 represent the alkaline hydrolysis ladder and T1 digestion ladder, respectively. B) Representative plot of the intensity of bands from nucleotides 36 to 45 for uncomplexed pre-miR-21 (black line), pre-miR-21 complexed with 2 μM streptomycin (gray line), and pre-miR-21 complexed with 5 μM streptomycin (dotted line). C) The sites of protection mapped to the pre-miR-21 structure (colored in gray, numbered in black).

the stem loop junction (protected residues G45, A42, C41, U40, and C39 are highlighted in gray and numbered in black in Figure 4C). Similarly, RNase T1 probing in the presence of 5 μM streptomycin showed a protection for the residues C39, G35, G32, and G28, spanning the region bound by streptomycin as determined by the docking study (see Figure S6).

To quantify the strength of binding of streptomycin to the pre-miR duplex we used the relative thermal stability (ΔT_m) approach as described by Pilch et al.[25] A truncated version of pre-miR-21 containing the terminal loop and a short stem having the bulge close to the terminal loop was used for CD melting experiments (from A23 to C52; see Figure S7B in the Supporting Information). The relative thermal stability in the presence of streptomycin was approximately 10 °C, which corresponds to a binding strength in the order of $10^7\,\text{M}^{-1}$. However, two structurally related aminoglycosides, amikacin and dihydrostreptomycin, showed no stability, thus indicating that they do not bind to the RNA (see Figure S7A). Fluorescence titrations were also carried out at 100 nM RNA concentration in 10 mM sodium cacodylate buffer containing 10 mM sodium chloride, and 1 mM magnesium chloride (pH 7.5) at 25 °C. Truncated pre-miR-21 was labeled with 2-aminopurine at the A7 and A20 (A29 and A42 in the pre-miR-21) positions, where protection were seen in presence of streptomycin. Titration of labeled truncated pre-miR (see Figure S8) with streptomycin showed a gradual increase in the fluorescence signal as streptomycin concentration was increased, whereas dihydrostreptomycin showed a minimal increment in the fluorescence signal. To calculate the binding affinity of streptomycin and dihydrostreptomycin to truncated pre-miR-21, relative fluorescence intensity changes were plotted against streptomycin and dihydrostreptomycin concentrations and fitted as described in the Supporting Information. The equilibrium association constants were obtained to be $(9.63 \pm 0.49) \times 10^6\,\text{M}^{-1}$ in the case of streptomycin and $(2.01 \pm 0.70) \times 10^5\,\text{M}^{-1}$ in th ecase of dihydrostreptomycin (see Figure S8). This data clearly indicates that streptomycin has around 50-fold higher affinity to pre-miR-21 than dihydrostreptomycin.

Having established that streptomycin binds to pre-miR-21 at a region close to the stem loop junction, we next asked how streptomycin represses the levels of miR-21. As indicated earlier the secondary structure of miRNA is preserved intact along the pathway of biogenesis. On the basis of our observations we considered the following possible mechanisms by which streptomycin could elicit the repression. To begin with, we imagined it could interfere with export of pre-miRNA by binding to any of the secondary structures along its length. However we ruled out this possibility since bulges along the stem are dispensable for export[26] and the binding region determined earlier is close to the stem loop junction.

The other mechanism we considered was inhibition of Dicer-mediated maturation of pre-miRNA by binding of streptomycin. To test this possibility we investigated the Dicer cleavage activity of pre-miRNA in the presence of streptomycin at different concentrations (from 2 μM to 5 μM; Figure 5). A significant decrease in the level of mature miR-21, compared to that of the untreated control, was observed and is lowest at 5 μM streptomycin. Two aminoglycosides

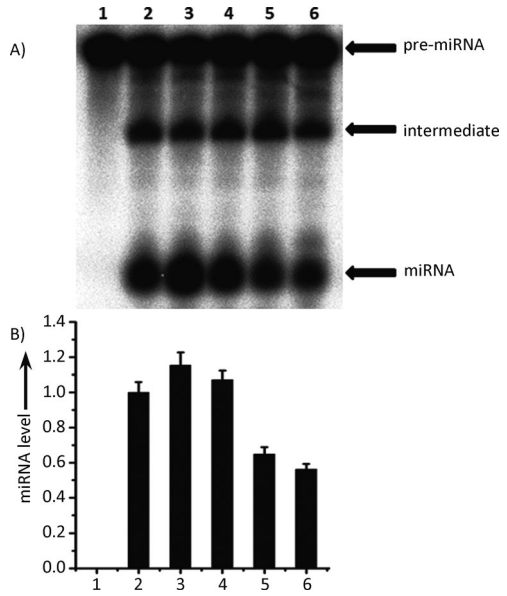

Figure 5. A) Dicer blocking assay (lane 1: pre-miR-21, lane 2: pre-miR-21 with Dicer, lanes 3 and 4: pre-miR-21 with Dicer in the presence of 5 μM amikacin and dihydrostreptomycin, respectively, lanes 5 and 6: pre-miR-21 with Dicer in the presence of 2 μM and 5 μM streptomycin, respectively). B) Densitometric analysis of miRNA levels for the above-mentioned assays showing a decrease in the amount of miR-21 formed in presence of an increasing concentration of streptomycin; amikacin and dihydrostreptomycin have no impact on Dicer processing.

having similar structures, amikacin and dihydrostreptomycin, were also taken as controls and were not able to inhibit Dicer cleavage. This experiment was also performed with pre-miR-103 and pre-miR-95 in the presence of 5 μM streptomycin, and they showed normal maturation levels (see Figure S9 in the Supporting Information). We concluded that the binding of streptomycin to the terminal loop region of pre-miR-21 might physically block Dicer access to the pre-miRNA. Alternatively it might induce structural rigidity leading to impaired processing of pre-miRNA as recent evidence suggests.[27] Unchanged levels of mature miR-103 and miR-95 also ruled out the possibility of direct binding of streptomycin to Dicer and blocking its action.

We probed the importance of the bulge near the terminal loop (as revealed by secondary structure analysis and protection assays) in the binding interaction of streptomycin and pre-miR-21. For this purpose, we used a mutant pre-miR-21 transcript where the bulge is absent (see Figure S10 in the Supporting Information). In vitro enzymatic foot printing did not show any protection as previously observed in presence of streptomycin (see Figure S11). No stabilization was observed (see Figure S12) when thermal melting was repeated with the mutant transcript and streptomycin. The single melting domain seems to be due to the removal of the bulge (see Figure S10). A Dicer blocking assay in the presence of streptomycin also did not show any effect in miR-21 maturation (see Figure S13). Collectively the evidence suggests that the bulge is necessary for the binding of streptomycin to pre-miRNA and hence the inhibition of Dicer processing. To check the effects in cellulo, we co-transfected pEZX-MT01 plasmid (expressing firefly luciferase reporter with the 3′UTR of PDCD4) and mutant pre-miR-21 in Jurkat cells. Streptomycin treatment did not show any significant increase in the luciferase signal compared to that of the untreated control. However, the same assay with wild-type pre-miR-21 showed a 1.2-fold increase in the luciferase signal in streptomycin-treated samples (see Figure S14). More importantly the overexpression of this bulge mutant in Jurkat, followed by treatment with streptomycin did not show any significant change in PDCD4 levels (see Figure S15). However, when the experiment was repeated with wild type pre-miR-21, the level of PDCD4 increased by 1.4-fold in the streptomycin-treated sample (see Figure S15). These observations, point towards the importance of the bulge in pre-miR-21 for efficient targeting by streptomycin in cellulo.

PDCD4, an apoptosis inducer thought to function by binding initiation factor EIF4a and causing a global translational arrest,[28] is one of the main targets of miR-21. We wanted to know whether miRNA-21 down regulation by streptomycin, and its consequent effect on PDCD4 (Figure 2), would have an effect on apoptosis and cell death. MCF-7, a breast cancer cell line was treated with streptomycin and the cell viability was determined by performing an MTT assay. We found that compared to the untreated control, there was a 25% reduction in cell viability in the streptomycin-treated cells ($p < 0.0001$; see Figure S16 in the Supporting Information). Flow cytometry using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen), was also performed to assay for cell death. Cells in the early stages of apoptosis would stain one of the two dyes, whereas cells staining for both are at end-stage apoptosis. The results (Figure 6) indicate that there is an increase in apoptosis (of approximately 2.5-fold compared to untreated) in streptomycin-treated cells, and is thus consistent with the effects of increasing levels of PDCD4 protein compared to those in the untreated control (Figure 2); the structurally related dihydrostreptomycin did not show any significant change. Additionally, the apoptotic distribution in Jurkat cells was fairly constant in both the cases (Figure 6).

Recently it was shown that miR-21 is a true oncogene, the depletion of which causes drastic tumor regression by

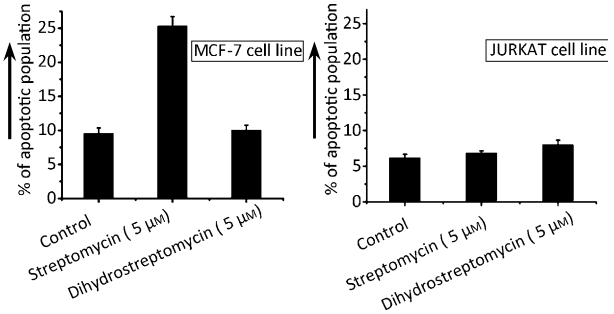

Figure 6. Results of a FACS analysis. Streptomycin treatment of the MCF-7 cell line caused around 2.5-fold increase in apoptotic population compared to untreated control, while dihydrostreptomycin did not show any significant effect. In the Jurkat cell line both streptomycin and dihydrostreptomycin had no effect.

apoptosis in an in vivo mouse model of pre-B-cell lymphoma, thus confirming previous reports.[29] Our results above indicate that streptomycin, a first line drug for the treatment of tuberculosis, can effectively inhibit miR-21 function and have similar phenotypic consequences at least in an in vitro cell culture model.

In summary, using a series of in vitro and cell-based assays, we show that streptomycin can efficiently repress miR-21 levels by direct binding to its precursor form and interfering with its downstream processing by Dicer. We also show that the inhibition by streptomycin is mostly specific for miR-21 as it fails to inhibit other related miRNA.

MicroRNAs are critical for many cellular processes, therefore deregulation of miRNAs can contribute to pathophysiology, including those of cancer. There is increasing evidence that inhibition of specific miRNAs in disease cells and tissues can induce favorable therapeutic responses.[30] The development of new ligand scaffolds will be important for generating a more diverse set of building blocks for the designing of small molecules that can inhibit miRNA function more specifically. This initial report paves the way for exploiting aminoglycosides as scaffolds for more effective inhibitory modules in general.

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