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Streptomycin, an inhibitor of miR-21 reduces invasion and suppresses tumor growth

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Micro RNAs (miRNAs) are small non-coding RNAs that post-transcriptionally regulate around 60% genes. These small RNAs play important roles in maintaining biological robustness and deregulation of miRNAs has been associated with different diseases, including cancer. Thus, modulation of miRNA levels is of immense therapeutic importance. miR-21, one of the important oncomirs over-expresses in different type of cancers and targets tumor suppressor genes, which are involved in invasion and metastasis. This makes miR-21 a potential therapeutic target. Previously, we have shown that streptomycin down-regulates miR-21 expression by hindering the processing of Dicer, an enzyme that facilitates the maturation of pre-miR-21 into its mature form in the cytoplasm. Here, we have explored the anti-cancer potential of streptomycin via modulation of miR-21. Our kinetic analysis shows the strong binding interaction between pre-miR-21 and streptomycin. In cell culture system, streptomycin treatment significantly reduces the invasiveness of B16F10 cells (where miR-21 is over-expressed). In mouse xenograft model, injection of streptomycin pretreated cells causes significant delay in tumor formation with reduced tumor size, when compared with untreated or other structurally related aminoglycoside treated cells. These results clearly indicate the therapeutic potential of streptomycin in reducing the aggressiveness in cancer.

Keywords: Amikacin, Cancer, Dihydro-streptomycin, Invasiveness, Tumor model

Micro RNAs (miRNAs) are one of the most important, evolutionarily conserved, non-coding RNA molecules that play crucial role in gene expression. They are transcribed as long primary transcript (pri-miRNA), which comprises several kilo bases and then are processed into a smaller (~70 nts) precursor (pre-miRNA) by the microprocessor complex. This hairpin intermediate (pre-miRNA) is then transported to cytoplasm via exportin 5-Ran GTP complex and is further processed by dicer enzyme, producing the mature duplex miRNA. The guide strand gets incorporated into RNA-induced silencing complex (RISC), followed by binding to the 3'-UTR (untranslated region) of their target mRNAs and regulates post-transcriptional gene expression via translational inhibition and/or mRNA decay[1].

Over the past two decades, researchers have explored the role of these miRNAs in different biological phenomenon. It is reported that around 60% genes are regulated by miRNAs[2]. These small non-coding RNAs have emerged as nodal regulators in different physiological and developmental processes[3-6]. Deregulation of miRNAs has been reported to be associated in different diseases, *viz.* metabolic disorders, cardiovascular diseases, neurological disorders, cancer, etc.[7-9]. Hence, modulating miRNA(s) expression in diseased condition is of great therapeutic importance[10]. To this end, oligonucleotide based methods (anti-miR, miRNA sponges, miRNA mimics, etc.) have already been explored *in vitro* and *in vivo*[11-14]. However, though they can be used as tools to modulate miRNA expression and thereby providing insight into its function, but the poorly understood pharmaco-dynamic and pharmacokinetic properties, lack of targeted delivery and unfavourable scalability and storage make them less preferable as therapeutic candidate[15]. Thus, development of small molecule modulators of miRNA expression is of prime importance[16]. Recently, small molecule modulators[17] of miRNA expression and function are being explored in the context of different diseases both *in vitro* and *in vivo*.

MicroRNA-21 (miR-21) is one of the most important oncogenic miRNAs and is reported to be upregulated in different types of cancers[18,19]. Recent studies have highlighted the importance of miR-21 as

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Abbreviations: AK, amikacin; DST, dihydro-streptomycin; SPR, surface plasmonresonance; ST, streptomycin.

diagnostic and prognostic marker for human malignancies[20], as well as its importance in mediating resistance against antibody-based cancer therapy[21]. miR-21 over-expression in cancer cells stimulates intravasation, invasion and metastasis by down-regulating tumor suppressor genes, namely programmed cell death 4 (PDCD4), tropomyosin 1 (TPM1) and maspin which are involved in tumor invasion and metastasis[22,23]. Thus, targeting miR-21 to modulate its expression and function is emerging as potential anti-cancer therapeutic strategy[24].

Our group and others have already reported small molecule inhibitors of miR-21[25-27]. While Gumireddy *et al.*[25] reported small molecule that can inhibit miR-21 by targeting the transcription of miR-21, we have shown that streptomycin, an aminoglycoside binds to pre-miR-21 and reduces the mature miR-21 formation by blocking dicer activity[26]. In this study, we have used surface plasmon resonance (SPR) based binding study to quantify association of streptomycin to pre-miR21 and then examined, if streptomycin-pre-miR-21 binding has any possible antitumor activity. We have evaluated the effect of streptomycin on invasiveness of cancer cells *in vitro* and tumor-forming ability of cancer cells *in vivo*.

Materials and Methods

Surface plasmon resonance (SPR) analysis

Pre-miR-21 and streptomycin binding studies were performed using a BIAcore 3000 instrument set at 25°C. Streptavidin coated sensor chip SA was purchased from BIAcore (GE Healthcare, Piscataway, NJ) and prepared using reagents from the manufacturer. Biotinylated pre-miR-21 was suspended in a buffer containing 10 mM HEPES, 10 mM NaCl and 1 mM $MgCl_2$ and injected over streptavidin conjugated flow cell until the desired surface density was reached. The adjacent cell was injected with the same buffer only to give blank control. Equilibrium binding experiments were carried out as follows. Streptomycin was injected (at flow rate 20 µL/min for 3 min) in concentrations ranging from 640 nM to 10 µM. A dissociation phase of 25 min ensued in the same running buffer. Regeneration was accomplished using 1 M NaCl in 50 mM NaOH for 30 s, followed by a 160 s stabilization period in running buffer at 30 µL/min. Experimental data were processed using BIA evaluation software version 4.1.1 (GE Healthcare). The sensorgrams for binding data were then fit to a 1:1 (Langmuir) binding interaction model to determine the rate constants (kon and koff) and the apparent equilibrium binding constant (K_A).

Cell culture

B16F10 (mouse skin melanoma) cell (mycoplasm free) was purchased from National Centre for Cell Sciences, Pune, India. Cells were cultured in antibiotic-free DMEM or 10% serum containing DMEM. Cultures of 85-90% confluency were used for all of the experiments. Cells were trypsinized, counted and seeded in 6-well plates for studies, and then allowed to adhere overnight before they were used for experiments. Thereafter, cells were treated with various drugs (streptomycin, dihydro-streptomycin and amikacin) at 5 µM concentration.

Invasion and migration assay

CytoSelect™ 24-well cell migration and invasion assay (8 mm, colorimetric format) kit (Cell Biolabs Inc., USA) was used to determine the invasive and migration properties of B16F10 cell in presence of different drugs: streptomycin (ST), dihydro-streptomycin (DST) and amikacin (AK). In case of invasion, the chamber plate was properly warmed up, followed by 1 h incubation by adding 300 µL of warm serum-free media (antibiotic free) to the inner compartment to rehydrate it. After removing the rehydrating media, cells were seeded at 2×10^5 cells/well and allowed to invade towards lower chamber containing 500 µL of serum containing media for 24 h in the presence or absence of 5 µM ST, DST and AK. The seeded cells were either untreated or pretreated with ST, DST or AK for 24 h at 5 µM concentration.

For migration studies, cells were seeded (either untreated or pretreated with 5 µM of ST, DST and AK for 24 h) at 2×10^5 cells/well and allowed to migrate toward serum containing media (antibiotic free) in lower chamber for 24 h in presence or absence of 5 µM ST, DST and AK. Invasive or migratory cells on the bottom of the membrane were stained and quantified at 550 nm after extraction.

Animal study

For this study, firstly, different sets of B16F10 cells were either kept untreated or pretreated with 5 µM of ST, DST or AK for 48 h. For allograft model, female C57BL6/J mice were obtained from NIN (Hyderabad, India). Mice, 6-8 weeks old were divided into four groups: mice injected with untreated B16F10 (UT) cells (4 mice), or mice respectively injected with ST, DST and AK-pretreated B16F10 cells (3 mice each). Tumor cells (either untreated or pretreated) were

implanted subcutaneously into the left flank of mice and left for observation up to 4 weeks. Tumor sizes were measured three to four-times in a week. Experiment was terminated because of one mouse death from untreated (UT) group.

Results and Discussion

Streptomycin binds to pre-miR-21 *in vitro*

In our previous study, we have shown that streptomycin down-regulates miR-21 by binding to pre-miR21 and thus blocking dicer activity[26]. We have also reported binding of streptomycin (ST) and pre-miR-21 via fluorescence titration method[26]. In order to have better understanding of pre-miR-21 and streptomycin interaction through binding kinetics, in this study we used surface plasmon resonance (SPR) analysis. SPR is one of the most powerful methods to measure real time binding kinetics of two interacting partners. Here, SPR was used to determine the binding parameters of pre-miR-21 and ST interaction (Fig. 1). The sonograms were analyzed using 1:1 (Langmuir) fit and the goodness of fit (measured by χ^2 value) was 0.0168. Binding affinity value of ST, K_a determined from the steady state region of sensorgram for pre-miRNA was 2.19 ($\pm$ 0.7) $\times$ 10^7 with association rate constant *k*a and dissociation rate constant k_d values of 1.49 ($\pm$ 0.2) $\times$ 10^5 M^{-1} s^{-1} and 6.80 ($\pm$ 0.5) $\times$ 10^{-3} s^{-1}, respectively. The binding affinity determined by SPR also matched with our previously published data[26].

The association and dissociation rate constants indicated the strong association and very slow dissociation of ST to the pre-miR-21. The strong affinity for ST to pre-miRNA-21 was mainly derived from the effect on dissociation kinetics, which is very slow in nature when we compare with other drug-nucleic acid interactions. Moreover, binding affinity was much stronger in the present case, when compared with binding of ST to the A-site of the decoding region of 16s RNA in the bacterial ribosome which is 1.06 $\times$ 10^6 M^{-1} [28]. There are several small molecule drugs, including aminoglycosides that show their drug ability by interfering with RNA function. Streptomycin is one of this class of molecules that functions by binding to the A-site decoding region on bacterial 16S ribosomal RNA[29]. The typical binding affinities of this class of molecules for their targets are in the micromolar range. In the present study, we also observed similar binding affinity of ST for pre-miR-21.

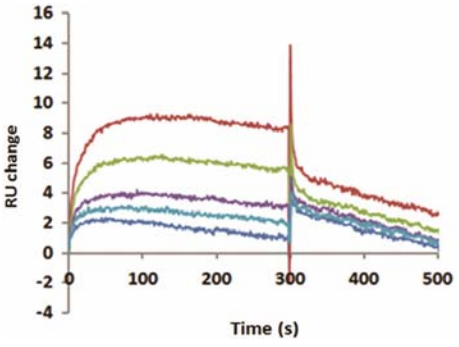

Fig. 1—SPR binding assay: SPR sensogram showing binding kinetics of streptomycin and pre-miR-21 interaction [The 5'-biotinylated pre-miR-21 was immobilized on to the SPR sensor surface (SA) and titrated with the streptomycin at varying concentrations (640 nM to 10 µM)].

It is well-known that aminoglycosides bind to many other different types of RNA structures, in addition to their pharmacologically relevant A-site decoding region target, such as regions of HIV mRNA[30,31] to thymidylate synthase mRNA[32]. This could be due to the fact that the RNA molecules that bind to aminoglycosides typically possess non-duplex structural elements[33,34] with asymmetric bulges or bubbles, which allow aminoglycoside access to the purine and pyrimidine bases[33-36]. Thus, in the present study, as pre-miRNA-21 also possesses non-duplex structural elements with asymmetric bulges or bubbles, it was targeted by ST with similar affinity.

Streptomycin reduces invasiveness of B16F10, but has no effect on migration

miR-21 is up-regulated in different solid tumors and helps in proliferation, tumor progression and metastasis[37]. In xenograft model, miR-21's temporal upregulation leads to tumor formation, whereas downregulation leads to tumor abolition in different organs[38]. However, metastasis, aggressiveness in tumor and progression in its size is partially attributed to invasiveness and migratory property of tumor cells. Towards this, we studied the effect of ST on cell invasion and migration using B16F10 mouse melanoma cell line, as miR-21 is reported to be upregulated in this cell. Moreover, B16F10 is a well-known melanoma cell line, which shows very high aggressiveness in mouse model partially due to both of its invasive and migration activity. Hence, invasion and migration studies were performed with this cell line using three different drugs (ST, DST, AK) with structural similarity.

B16F10 cells were kept untreated or pre-treated with ST, DST and AK. ST-treated B16F10 cells

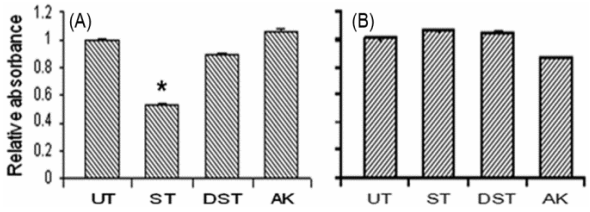

Fig. 2—Invasion and Migration assay: Colorimetric analysis of invasion (A) and migration assay (B) of B16F10 cell line [Cells were either kept untreated (UT) or treated with streptomycin (ST), dihydro-streptomycin (DST), or amikacin (AK). The asterisk * denotes $P < 0.05$ while comparing with UT]

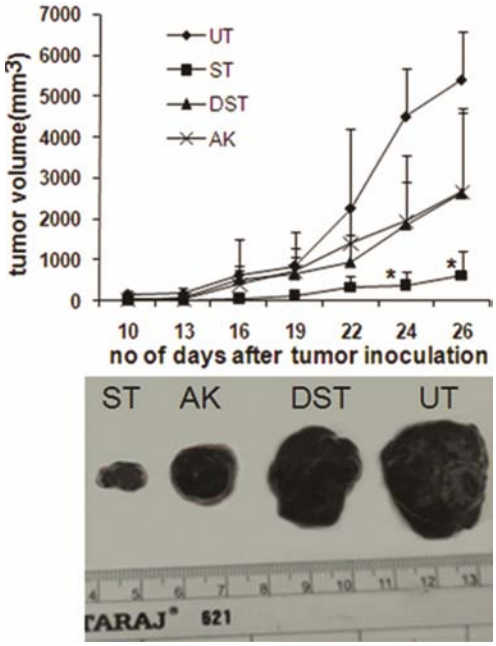

Fig. 3—*In vivo* tumor growth study: Tumor growth curve (upper panel) after pre-treatment of different drugs (5 µM, 48 h) in B16F10 cells, followed by subcutaneous implantation in C57BL6/J mice [Cells were either kept untreated (UT) or treated with streptomycin (ST), dihydro-streptomycin (DST), or amikacin (AK). The asterisk * denotes $P < 0.05$, while comparing with UT. The image picture (lower panel) exhibits the representative sample of B16F10 tumors excised after sacrificing mice on day 26]

showed significant reduction in invasiveness *in vitro*, while DST or AK-treated cells exhibited no change in aggressiveness. However, except for AK, which exhibited certain mild effect, ST and DST had no effect on migration of B16F10 cells (Fig. 2). This data clearly indicated that ST had the inhibitory effect on invasiveness, but not on the migratory activity of these cancer cells.

Streptomycin induces tumor growth inhibition

After the first report of development of small molecule inhibitors for miR-21 m-RNA expression[25], many new small molecule-based anti-miR-21 strategies have been developed. Shi *et al*.[37] reported a small molecule inhibitor of miR-21 that eventually suppresses tumor growth. We also showed recently that ST could strategically bind to pre-miR-21 to down-regulate miR-21[26]. However, in order to see the direct effect of ST on tumor forming ability of treated cancer cells, we inoculated tumor cells in mice. Generally, following subcutaneous inoculation of cancer cells in mice, tumor start to grow and become very aggressive, leading to increase in effective size of tumor. For exhibiting aggressiveness, the tumor cells have to invade the extracellular matrix (ECM) for which the cells should have potent invasiveness[39].

As ST induced potent reduction of invasiveness in B16F10 cells, we next examined if this pre-condition was subversive for proper tumor production and growth or not. Thus, B16F10 cells were either pre-treated with ST, DST and AK at 5 µM concentration or kept untreated for 48 h. Subcutaneous implantation of ST-pretreated cells produced significantly small sized tumors, whereas the untreated and DST and AK pre-treated B16F10 cells produced large tumors. This indicated that ST pretreated B16F10 cells were less aggressive for tumor formation (Fig. 3) and hence this could be related to reduction in invasive property of ST-treated B16F10 cells.

In conclusion, the present study demonstrated the antitumor effect of streptomycin, which showed potent binding to pre-miR-21, thereby inhibiting the miR-21 processing. This may pave way to design more effective therapeutic molecules using streptomycin as scaffold.

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