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Gene Expression Analysis in MOTN-1 Cell Line after Treating with New Development Aptamer

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ABSTRACT

Among age-related morality in humans, cancer stands at 2nd leading cause and this is why the ability to suppress carcinogenesis has got the cancer prevention and treatment researchers working hard on this. Leukemia (cancer of bone marrow and blood), is most commonly characterized by uncontrolled proliferation of some blood cells, in this case leukocytes. There are generally four types of leukemia: acute myelogenous, chronic myelogenous, acute lymphocytic and chronic myelogenous. There are quite a few different leukemia cell lines and among them, the MOTN-1 cell line was derived from acute lymphoblastic leukemia. Moreover, the MOTN-1 cells are commonly described a highly undifferentiated leukemia cells. In this study, we evaluated the effects of SIRT1 activation by aptamer (Aptamer are single strand DNA or RNA molecules, selected by an iterative process known as Systematic Evolution of Ligands by Exponential Enrichment SELEX) on viability of MOTN-1 cancer cell line as an anticancer drug and analysed the gene expression profiles of MOTN-1 cells treated with aptamer and untreated cells by cDNA microarray analysis and RT-PCR to give us more details about the molecular mechanisms underlying aptamer-induced carcinogenesis, including leukaemia. The results demonstrate that aptamer is extremely efficient tools for anticancer activity on ALL by inhibited 91% of these cancer cells at 10 µM aptamer after 72 hours with IC_{50} = 3.5 µM. Besides that, we at first identified 13 genes with expression changed by aptamer treatment. For this set of genes, 11 of these were up-regulated and two were down-regulated by more than 3-fold, respectively. As a result, pharmacological activation of SIRT1 enhanced cell death suggesting a tumor suppressive function of aptamer and may be used in the future for cancer treatment.

Keywords: Microarray, Aptamer, Cytotoxicity, Gene expression, Acute Lymphoblastic Leukaemia.

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INTRODUCTION

Leukemia, one of the most lethal malignancies worldwide (1), is a heterogeneous group of hematopoietic cancers with more than 61,780 estimated cases to be diagnosed with leukemia and 22,840 estimated deaths in 2019 in the United States (1). Generally, there are 4 categories of leukemia including acute lymphoblastic leukemia (ALL), acute myeloid leukemia, chronic lymphoblastic leukemia, and chronic myeloid leukemia. Notably, great efforts have been dedicated against leukemia leading to the making of new anti-leukemic therapies with higher efficacy and less unspecific actions. This also includes advancement in delivery methods such as encapsulation, both control release and targeted delivery of cytotoxic therapies into the cancer sites (2). Indeed, the only prevalent approaches for leukemia treatment are bone marrow transplantation and chemotherapy. However, these types of interventions have a relatively narrow spectrum compared to those which are available for solid tumors with a non-specific delivery while the chemotherapy has serious side effects including myelosuppression, cardiotoxicity, leucocytosis, as well as alopecia (3).

The normal blood cells surround leukemia cells, which is why these drugs do not differentiate between pathological cells and normal cells. So, this is how toxic materials are able to affect normal blood cells. Too much exposure of normal cells to these kinds of drugs commonly leads to the use of a suboptimal dosage. This is the culprit behind many of the toxicities associated with these kinds of drugs. Together with the appearance of drug-resistant pathological cells, there is an urgent need for a targeted and potent approach which should be selective and efficacious therapies with minimal toxicity to specifically kill the malignant cells only without affecting other normal blood cells (4, 5).

Sirt1 belongs to the family of sirtuins (in mammals). It is a protein called NAD-dependent deacetylase or Nicotinamide Adenosine Dinucleotide dependant deacetylase. Its function is to remove acetyle groups from both types of proteins called non-histone and histones. Due to its deacetylating abilities it can deacetylate a large number of substrates. This deacetylating property makes it a broad range protein and is involved in many physiological functions. These physiological functions include – Controlling the expression of genes, controlling the process of aging and metabolism. (6, 7) Sirt1 in cancer activates the tumour suppression, DNA damage repair, genomic stability, activates the deacetylation of β-catenin, epigenetic silencing of DNA hypermethylated TSGs and sirt1 inhibits the transcriptional activity of p53, and c-myc stability. (8, 9) SIRT1 is gaining popularity as a target for the developments of drugs especially for the treatment of disease which are age related, or cancer etc. Resveratrol is a natural sirt1 activator compound, it has proven to be beneficial for controlling tumorigenesis, obesity and other age related health problems. These problems include neuronal loss and decreased cardiac function. Another recently discovered Sirt1 activator is endogenous AROS. IT increases the deacetylating capacity of SIRT1 especialy towards p53 by binding to the N-terminal of SIRT1.(10) The attachment of small ubiquitin related modifiers or the process of sumoylation also potentiates the deacetylating properties of SIRT1. SUMOs attach to the lysine 734 and activates SIRT1 which ultimately regulates the cellular

reponse towards genotoxic stress.(11) However, there is a conflict about the role of SIRT1 in cancers. Some studies have shown increased SIRT1 in different cancers while others found down-regulated or unchanged SIRT1 levels in cancers. Another study showed that there are high levels of SIRT1 in the mucosa of colon and also in adenomas which are benign in nature. In almost 25% of the I, II and III stages of colorectal adenocarcinomas, SIRT1 was found to be overexpressed. However, in stage IV cancers it was rare.(12) On the contrary, from samples collected from patients suffering from lung cancer, colon carcinomas, leukemias etc, SIRT1 was found out be overexpressed and increase.(13, 14) This might make the researchers believe that its inhibition not activation that results in the suppression of tumour proliferation. However, in cancers like breast cancer or liver carcinomas the levels of SIRT1 was reduced. In cancers like lung, skin, bladder etc. there was no change in the levels of SIRT1 and slightly raised levels of SIRT1 were observed in thyroid cancers. This urges the researchers to put in more effort to understand the true mechanism by which SIRT 1 ca be used to control cancer. This study focuses and contributes to the unclear data of how SIRT1 activators like aptamers can control tumour growth.(15)

Aptamers are artificial short, oligonucleotides comprising RNA, DNA or peptide aptamers with specific secondary and tertiary structures, which exert their biological and physiological effects by binding to targeted proteins with a high binding affinity (16), and stability (17), versatile chemical modification (18), quick chemical production (19), better compatibility (20), and specificity (16).

The major strategy behind using the aptamers as therapeutics in the field of oncology is their ability to antagonize the protein-protein binding or receptor-ligand binding of cancer causing proteins. Anti-VEGF aptamer called pegaptanib shows anti-cancer activities by inhibiting the tumour vessels formation. Anti-EGFR aptamers such as CL-4 and E07 blocks the phosphorylation and the process of downstream signalling and is effective in treatment of vulvar carcinoma, lung cancer and breast cancer.(21)

Aptamers are selected and generated through an *in vitro* molecular method called systematic evolution of ligands by exponential enrichment (SELEX) which is a well-established and efficient technique for the screening of oligonucleotides with a high affinity for their targets from random-sequence libraries (22) with the ability to identify and screen of different aptamers (23). Though simple but highly effective, targered drug delivery system are made of therapeutic materials that go through covalent or noncovalent conjugation for the delivery of targeted agent. Targeted drug delivery systems have an amazing way of improving the efficiency of delivered drug agents for a specified area. Besides providing a substitute delivery mechanism, they can also enhance the accumulation in the cellular of diseased tissue. (24), aptamers are greater ligands accelerating the growth of aptamer-targeted drug delivery systems because they have low immunogenicity and toxicity, easily modified chemical structure, and a high specificity towards a wide range of targets, including antibiotics, DNA, RNA, glucides, viruses, toxins, ions, phospholipids, proteins, and even entire cells as well as tissues. Aptamer-targered drug delivery systems can be classified into 2 main categories, depending upon their unique compositions and preparation methods. These include aptamer-nanomaterial conjugated systems and aptamer-small molecule conjugated systems. (25). When compared with the conventional antibodies, aptamers are relatively greatly stable at even higher ranges of pH, ionic environment and temperature etc. They can be rather easily modified with several tags and have a higher half-life. (26-29). Since aptamers are smaller than antibodies in size, it leads to faster and more aptamers internalization into tumor locations.

Indeed, cancer cells levels are very low at the early stage of carcinogenesis in different body sites, hence, discovering of highly sensitive diagnosing and imaging techniques is greatly needed. Since aptamers could be produced through the expressional system or chemical synthesis (30) and modified easily, thence aptamers can be modified with fluorescent groups and used for diagnosing different cancers at earlier stages. Several articles demonstrated that RNA or DNA aptamers could be used in imaging tumor cells and tracking them *in vivo* as well as *in vitro* (30). Due to these great features, applications of aptamers are developing fast in not only imaging and diagnosing but also treating different cancers (30) such as breast cancer (31), glioma (32), prostate cancer (33), renal cell (34), hepatocellular (35) as well as ovarian carcinoma (36) and leukemia (3, 37, 38). Indeed, it is possible to detect and diagnose cancer (34, 37, 38) and infectious diseases (39, 40) in their early phases using the aptamers. A lot of research projects have shed light on the benefits and applications of aptamers (31, 38, 41-46) because of their unique characteristics. Aptamers can be easily modified and conjugated to several materials including imaging agents, siRNAs, as well as therapeutic drugs. Moreover, different aptamers have been selected against leukemia such as sgc8 aptamer which could specifically detect and internalized into ALL cells (47, 48) or AS1411 aptamer, a therapeutic aptamer, which is currently undergoing clinical trials for treating ALL (42, 45). This should be taken into account that aptamers are in great demand nowadays and are vital for research and development. Also, for targeted drug delivery systems, they are are critically beneficial especially in the case of drug delivery to tumors. Thanks to that, FDA (Food & Drugs Authority) have started approving some of the drugs for curing specific kinds of cancers. (49, 50).

In this study, we aimed to evaluate the effects of SIRT1 activation by aptamers on viability of MOTN-1 cancer cell line as an anticancer drug and analysed the gene expression profiles of MOTN-1 cells treated with aptamer and untreated cells by cDNA microarray analysis to give us further details about molecular mechanisms underlying aptamer-induced carcinogenesis, including leukaemia.

Materials and Methods

Materials

Chemicals were purchased from ThermoFisher Scientific, Sigma-Aldrich or SLS. Recombinant human SIRT1-GST-tagged (SIRT1-462H) was purchased from Creative BioMart. PreScission Protease on-column GSTrap FF and NHS-HP SpinTrap column were purchased from GE HealthCare. DNA library 5`-TTCGGAAGAGATGGCGAC-N40-CGAGCTGATCCTGATGGAA-3` was purchased from TriLink Bio Technologies. BAS-P1, BAS-P3 and BAS-P3-notail primers (BAS-P1: 5`-TTCGGAAGAGATGGCGAC-3`, BAS-P3: 5`-ATGTCGTGCGTGCTA-SP18-TTCCATCAGGATCAGCTCG-3` and BAS-P3-notail: 5`-TTCCATCAGGATCAGCTCG-3`) were purchased from IDTDNA. ISOLATE II PCR and Gel kit, VENT DNA polymerase, Vector Ptz57R/T and E. coli DH5α were purchased from Biolabs. Nucleospin® Extract II kit and QIAprep Spin Miniprep Kit were purchased from Qiagen.

Cell Lines and Cultures

MOTN-1 cells were purchased from ATCC and cultured in RPMI-1640 supplemented with 5% FBS, 1% L-Glutamine and 1% Penicillin-Streptomycin-Amphotericin B. Cells were cultured in 75 cm^2 flasks and incubated in 5% CO_2/95% humidified air at 37°C.

SELEX Approach to select aptamer against SIRT1

Human recombinant SIRT1, GST-tagged was cleaved by PreScission Protease on a GSTrap FF column following manufacturer's recommendations. The extent of cleavage of the SIRT1 enzyme was determined by SDS- PAGE. SIRT1 (1.0 mg/ml in coupling buffer) was immobilized in an NHS-HP SpinTrap column (1ml) following manufacturer's recommendations. For the initial round of selection, 2.5 µl (1000 pmol) BAS library were added to 200 µl of binding buffer (pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$) in the column containing SIRT1 and incubated for 4h at 37°C. Wash and elution buffers were formulated with 5mM $MgCl_2$ but differing NaCl concentrations ranging from 0.15 M to 1.5 M (all at pH 7.4 and 5mM $MgCl_2$: a-150mM NaCl; b-300mM NaCl,; c-600mM NaCl; d-900mM NaCl; e-1500mM NaCl). A wash step with 5 columns (1 ml) volumes of binding preceded the elution step. The product precipitated from the supernatant with 500 µl of 100% cold ethanol and was spun at 21,000g at 4°C for 30min. The pellet was washed with 70% ethanol and repeated the centrifugation step then dried and re-suspended in 20 µl water and set up PCR in 100 µl volumes. For the PCR **,** 1 µM of both BAS-P1 and BAS-P3 primers plus recovered sequences from the previous step, dissolved in 20 µl were used for the of PCR amplification process. The 18 atom hexaethyleneglycol spacer in the antisense primer BAS-P3 connects the complementary primer region to a 15 nt random sequence helping with strand separation of the PCR products by PAGE. The PCR programme 20 cycles run as: denaturation 95 °C 30 seconds, annealing: 50 °C 45 seconds and extension: 72 °C 10 sec, on a Robo Cycler Gradient 96 (Stratagene). The product was purified by urea PAGE and ISOLATE II PCR and Gel kit. Around 12 rounds were repeated for all the steps of selection aptamers as shown in Figure 1. The final population of the selection step, dissolved in 2 µl was used as a template, it was added 200 µM dNTP, 1 µM each of BAS-P1 and BAS-P3-notail primers, buffer and VENT DNA polymerase. PCR products were purified for the cloning using the Nucleospin® Extract II kit. The ligation reaction was mixed with 0.52 pmol of purified PCR product and added to 6µl of 5X ligation buffer, 0.17 pmol of Vector Ptz57R/T and 3U T4 DNA ligase in a final 30µl volume, stirred for 5 minutes and then kept on 4°C overnight. *E. coli* DH5α high-efficiency competent cells were used for transformation following the manufacturer's recommendation. For transformation, 100 µl cell cultures were plated onto duplicate LB/100 µg/ml ampicillin 50 µg/ml/IPTG/X–Gal plates. Colonies were incubated overnight at 37°C with gentle shaking, picked and inoculated into LB media supplemented with 50 µg/ml ampicillin and incubated overnight at 37 °C with shaking. QIAprep Spin Miniprep Kit manufacturer's recommendations were used for plasmid preparation, for each clone, 0.6 µg purified plasmid DNA was mixed with 20 pmol forward primers in a 100 µl PCR tube. All aptamer samples were outsourced for sequencing to Source BioScience. The Data of sequencing were analyzed by DNAMAN 5.29 software (Lynnon corp).

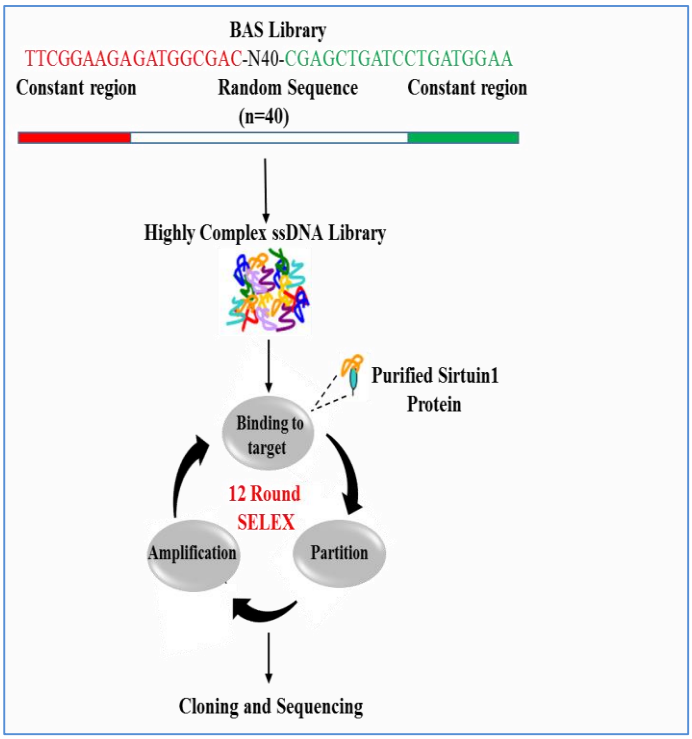

Figure 1. Scheme of selection of linear aptamer against SIRT1.

Cell Maintenance

MOTN-1 cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Fisher, UK) supplemented with 10% fetal bovine serum FBS (Fisher Scientific, USA) and 1% L-Glutamine (Lonza, UK) as well as to 1% Penicillin-Streptomycin-Amphotericin B 100X (Lonza, UK) as antiseptic. Cells were cultured in 75 cm^2 flasks and incubated in 5% CO2/ 95% humidified air at 37° C. Once the cells reached 90% confluency, flasks containing MOTN-1 cells were passaged under sterile conditions. The cells were washed with 5 ml of phosphate buffered saline solution (PBS) and then incubated for 2 minutes in trypsin solution at 37° C to let cells to detach themselves from the bottom of the flask. We added an equal volume of complete growth media before transferring the cell suspension into a 50ml conical tube. Cells were then centrifuged at 1200 rpm for 3 minutes. We discarded the supernatant and the cell pellet resuspended in fresh supplemented growth media. We then counted the cells under the microscope on a hemocytometer and used as required.

Cell Viability and Inhibitory Concentration (IC_{50}) by MTT Assay

The MTT assay was used to assess the effects of aptamer on cancer cells viability. A 100 µl from all cells suspensions (MOTN-1) were dispensed into 96-well flat-bottom tissue culture plates (Falcon, USA) at concentrations of 5 ×10^3 cells per well and incubated 24 hours under standard conditions; 4 × 10^3 cells/well for 48h incubation, and 3 × 10^3 cells/well for 72 hours incubation. After 24 hours, the cells were treated with (0.15, 0.312, 0.625, 1.25, 2.5, 5 and 10 µM) of the aptamer. After a recovery period 24, 48, and 72 hours, the cell culture medium was removed, and cultures were incubated with medium containing 30 µl of MTT solution (3 mg/ml MTT in PBS), (3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium Bromide) for 4 hours at 37° C. After 3h this medium was removed by

gentle inversion and tapping onto paper. Control wells received only 100 µl growth media.100 µl of dimethyl sulfoxide (DMSO) was added to each well, the plates were then kept at room temperature in the dark for about 15-20 min. The absorbance of each well was measured by a multiscan reader at a wavelength of 540 nm and correcting for background absorbance using a wavelength of 650 nm. The cells' viability was determined according to the optical density (OD) of the wells which contained no circular aptamer. The inhibitory concentration 50% (IC_{50}) was defined as the minimum concentration of the circular aptamer that reduced the viability of the incubated cells after 72 hours by 50%.

RNA Extraction

Trizon Reagent (Life Technologies, Rockville, MD) was used to extracted Total RNA and it was treated with RNAiso plus (Takara, Seoul, Korea) in accordance with the manufacturer's recommendations. The quality and integrity of the RNA were checked using 1% agarose gel electrophoresis, OD 260/280 ratio measurements, and analysis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA, USA).

cDNA Array and Data Analysis

Atlas Human Hematology cDNA expression array (No. 7737-1) was used in order to analyze gene expression of MOTN-1 cells. We found 406 duplicate spots showing cDNAs in each filter (only of known and sequence-verified genes. Clontech for more info on genes). Furthermore, we used Clontech cDNA array labelling kit for the purpose of converting total RNA (3 µg) into cDNAs. Later on, the filters were washed as per the guidelines of the manufacturer before treating with 10 µM aptamer. We then exposed the filters to imaging plates (BAS-MP 2040S; Fuji, Kanagawa, Japan) and afterwards they were scanned with a PhosphorImager (Bio-Imaging Analyzer, BAS2500; Fuji). Atlas Image 2.01 – a software by Clontech was used in order to check the intensities of the spots. This was helpful in comparing the intensity of each spot on array of MONT-1 with 10 µM aptamer with that of the corresponding spot on the MOTN-1 array after treatment with aptamer. For every gene, we quantified the intensity ratio and the results showed comparative abundance of MONT-1 without circular aptamer gene as compared to that in MOTN-1 with circular aptamer gene expression. We used global normalization – taking the mean value of all the genes to normalize the array. This was done in order to avoid any and every misinterpretation between the arrays that are being compared. We used both the sum and average methods in order to calculate the normalization coefficient. This, eventually got the same results as shown in the Atlas Image 2.01 user manual and also as in Atlas cDNA Expression Arrays (PT3140-1; Clontech).

Real-Time Quantitative RT-PCR

Once the MOTN-1 cells were treated by using 10 µM aptamer, the extraction of RNA from cells and generation of cDNA was done using 2.0 µg of RNA (Bioneer, Korea). The following primers were used for each of the genes: Dek, forward, 5'- GAAGAATGTGGGTCAGTTCAGTGGC-3', reverse, 5'- GGACATTTGGTTCGCTTAGCCTTCC-3'; rac, forward, 5'-GACGGAGCTGTAGGTAAAACTTGC-3', reverse, 5'- CAAATGATGCAGGACTCACAAGGG-3'; Op18, forward, 5'- CCCTCCTGGTTGATACTTGTTCCAG-3', reverse, 5'-GACATGCCCCACCTGTAACGTAGAG-3'; CD6, forward, 5'-GTACCCATCGGAGGCCAAGGTGC -3', reverse, 5'-GTGGACAGATTGTGCAAACTCCGGG -3'; CD58, forward, 5'-AATAGGGTTTATTTAGACACTGTGTCAGG -3', reverse, 5'-TTGAGTTACGTTTACATTGCTCCATAGG -3'; CD106, forward, 5'- TCGAGATGAGTGGTGGCCTCGTGAAT -3', reverse, 5'- GGAAAGCCCTGGCTCAAGCATGTCATAT -3'; Id2, forward, 5'-GAAAGCCTTCAGTCCCGTGAGGTCCGTT -3', reverse, 5'-CTGGTGATGCAGGCTGACAATAGTGGGATG -3'; ATF4, forward, 5'- AATGGCTGGCTGTGGATGGGTTGGTCA -3', reverse, 5'-GATCATGGCAACGTAAGCAGTGTAGTCTG -3'; IRF5, forward, 5'- CCAGTGACAAGCAGCGCTTCTACAC -3', reverse, 5'- TCTGGCCCTTTTGGAACAGGATGAG -3'; ELL2, forward, 5'-TACAGTGAAATAGACAGACGGTCATTGG -3', reverse, 5'-CCATTTAGTGTTGGTGGTACTCTGTTC -3'; D6, forward, 5'-CCTGCTCCTTGCTACCATAGTATGG -3', reverse, 5'-CACCAAGACACAACCAATACGGGAG -3'; GzmA, forward, 5'-GGAGGACTCACAATAGTGCATCTTGG -3', reverse, 5'- GAAGAATATAGACACCAGGCCCACG -3'; GzmK, forward, 5'-AGAAGTCACTGTTACTGTCCTAAGTCG -3', reverse, 5'- TTGTAACTTAATTTGTATGAGGCGGGAC -3'. The PCR reaction consisted of DNA Master SYBR Green I mix (Bioneer-Fast Start DNA Master SYBR Green I kit; containing Taq DNA polymerase, dNTP, 3 mM $MgCl_2$, and SYBR Green dye), 0.5 µM each primer, and 20 ng of cDNA.

The SYBR Green I dye has a unique way of acting as it gives fluoresces when binded in minor groove of double-standard DNA. However, during the annealing PCR phase, the fluoresces of amplified PCR fragments is sensed by the Light Cycler. To kick off the run, initial denaturation at 95 °C for 10 minutes was used, then 40 cycles of denaturation was given at 95°C for 15 seconds. The last two steps included annealing and elongation, aneealing was carried out for 10 seconds at a temperature between 56-60°C for and elongation was carried out for 15 seconds at a temperature of 72°C. To crosscheck the specificity of amplification melting curve analysis was combined with another process involving denaturation at about 95 °C and the starting and ending temperstures were 65 °C and 95 °C respectively. The rate of increase of temperature was set at 0.1 °C/sec. For every assay, a negative control without any cDNA template was performed concurrently. Serial dilutions of the β-globin gene (Bioneer-Control Kit DNA) were used to obtain standard curves to get relative concentrations. The supplier's instructions were followed closely in each attempt. Only those values were considered as suitable for quantification that were less than 0.3. Kinetics approach using the Bioneer software was used to get the relative concentration of each gene. The whole experiments was replicated and performed thrice. In order to make sure that the obtained SYBR signal was the actual product of PCR and of expcted size, all of the PCR products were run on agarose gel 2%.

Results

SELEX, DNA Cloning, and Sequencing

A workflow representation of the selection process leading to the discovery of SIRT1 DNA aptamers is shown in Fig. 1. A pool of ssDNAs was incubated with SIRT1 immobilized in a column. Oligonucleotides, in binding buffer, interacted with SIRT1 and weak interactions were disrupted by a range of buffers with an increased ionic strength. Bound oligonucleotides were recovered using a high salt step to disrupt the association with SIRT1, as shown in Fig. 2 desalted and amplified by PCR. Each round

was monitored for enrichment of binding aptamer, as shown in Fig. 3 in where a band at around 75 bp is getting enriched through the rounds. After 12 rounds of selection, the PCR products were cloned and 72 clones were obtained for consensus sequence family analysis; from them, 50 complete sequences were used in class analysis sorted, aptamer represented 28% of a total of 50 sequences. The DNA sequence of the aptamer is 5′ CACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTA 3′.

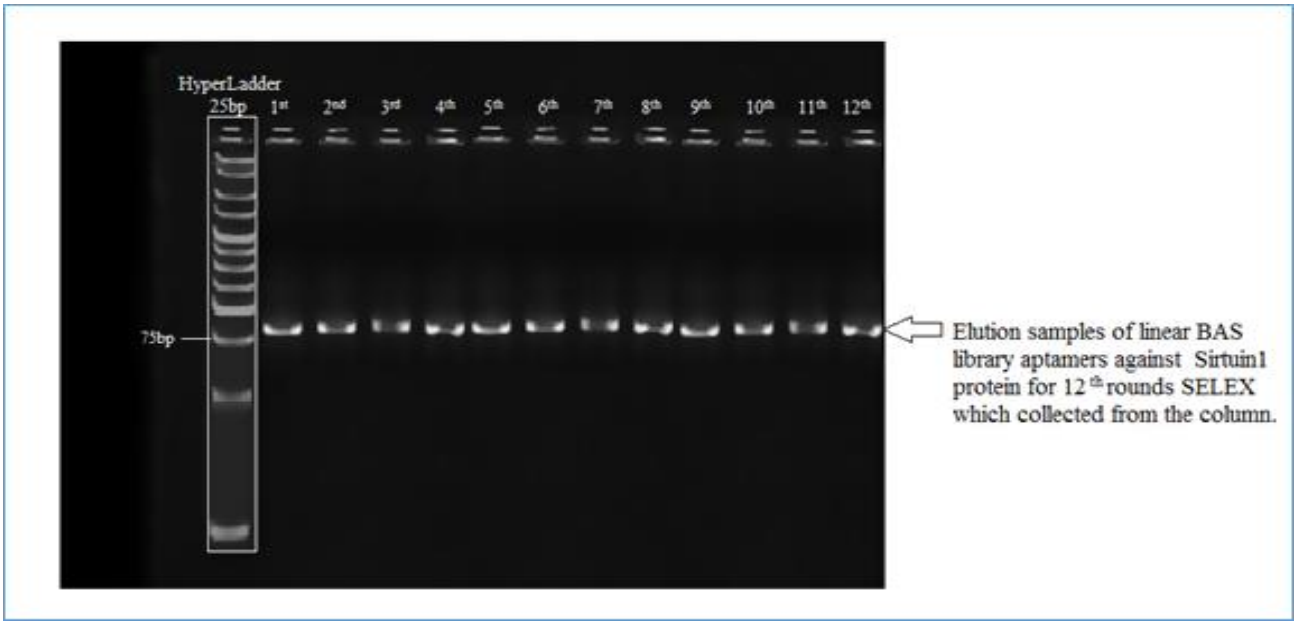

Figure 2. 10% acrylamide gel analysis of linear elution aptamer of *in vitro* selection against SIRT1 enzyme for 12th rounds SELEX before PCR.

Lane 1, HyperLadder 25 bp; Lane 2, the elution of bound BAS library after 1st washed; Lane 3, the elution of bound BAS library after 2nd washed; Lane 4, the elution of bound BAS library after 3rd washed; Lane 5, the elution of bound BAS library after 4th washed; Lane 6, the elution of bound BAS library after 5th washed; Lane 7, the elution of bound BAS library after 6th washed; Lane 8, the elution of bound BAS library after 7th washed; Lane9, the elution of bound BAS library after 8th washed; Lane10, the elution of bound BAS library after 9th washed; Lane 11, the elution of bound BAS library after 10th washed; Lane 12, the elution of bound BAS library after 11th washed; Lane13, the elution of bound BAS library after 12th washed.

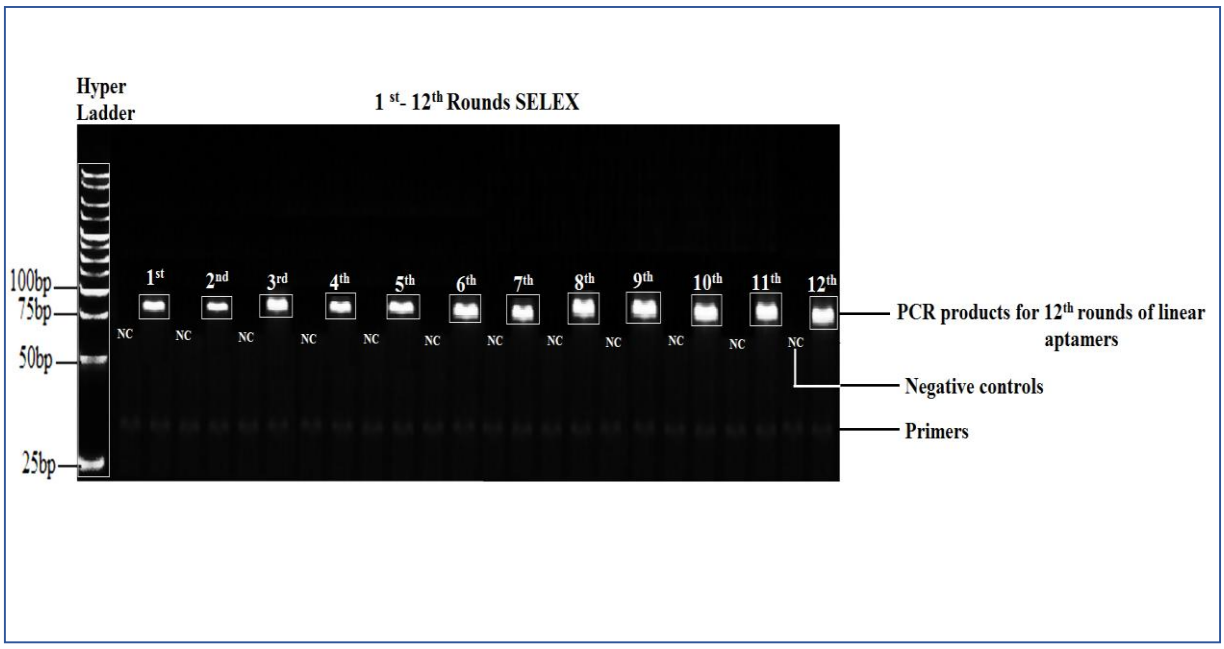

Figure 3. 10% acrylamide gel analysis of PCR products aptamers *in vitro* selection against SIRT1 enzyme for 12th rounds SELEX.

Lane1, HyperLadder 25 bp; Lane 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 are negative controls (NC). Lane 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25 are the PCR products of the 1st to the 12th round SELEX.

Binding for SIRT1 and Aptamer

Surface Plasmon Resonance (SPR) provides information on the interaction of a protein and its substrate; utilizing this method, aptamer was examined. The sensor surface was built up by immobilization of aptamers on the sensor chip, and SIRT1 was injected at different concentrations up to 800 nM. An aptamer level of 1000–1200 RU on the sensor surface was adjusted in this experiment over the injection time during the immobilization of the aptamer. The sensorgram (Fig. 4) revealed a very tight and stable binding behaviour of SIRT1 enzyme to the immobilized Aptamer. We obtained the constant of dissociation, K_D as 48.3 ± 0.986 nM.

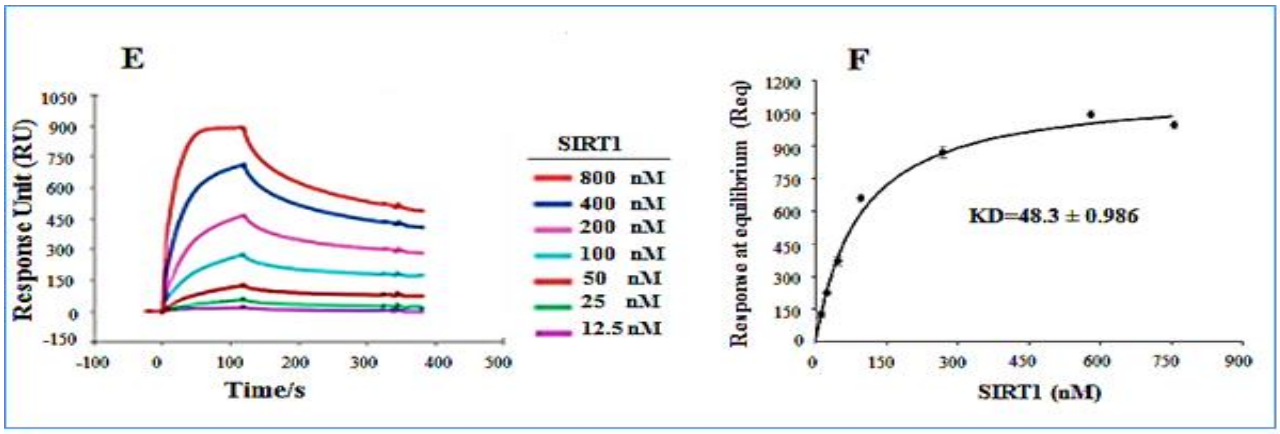

Figure Error! No text of specified style in document.**.** Determination of the affinities of aptamers for SIRT1 using surface plasmon resonance. (E) Sensorgrams of the binding response to aptamer measured for concentrations of 12.5, 25, 50, 100, 200, 400 and 800 nM SIRT1.

The KD = 48.3 nM as determined from a global fit of the kinetic simultaneous ka/kd model, assuming Langmuir (1:1) binding, and x^2= 0.986, (F) Plot of the steady-state affinity for `E' using the Req values derived from sensorgrams in (E) fitted locally.

Percentage of Cell Death of MOTN-1 Cell Line

To estimate the effect of linear aptamer on MOTN-1 cells viability, MOTN-1 cells were treated with (0.15, 0.312, 0.625, 1.25, 2.5, 5 and 10 µM) aptamer at 24, 48 and 72 hours (Fig. 5) p <0.005. Aptamer significantly increased the cells death of MOTN-1 cells at 10 µM (51, 83 and 91%) at 24, 48 and 72 hours respectively p <0.0001 and 5 µM (38, 43 and 81%) at 24, 48 and 72 hours respectively p <0.0005 versus other concentrations as illustrated in Figures 6 and 7.

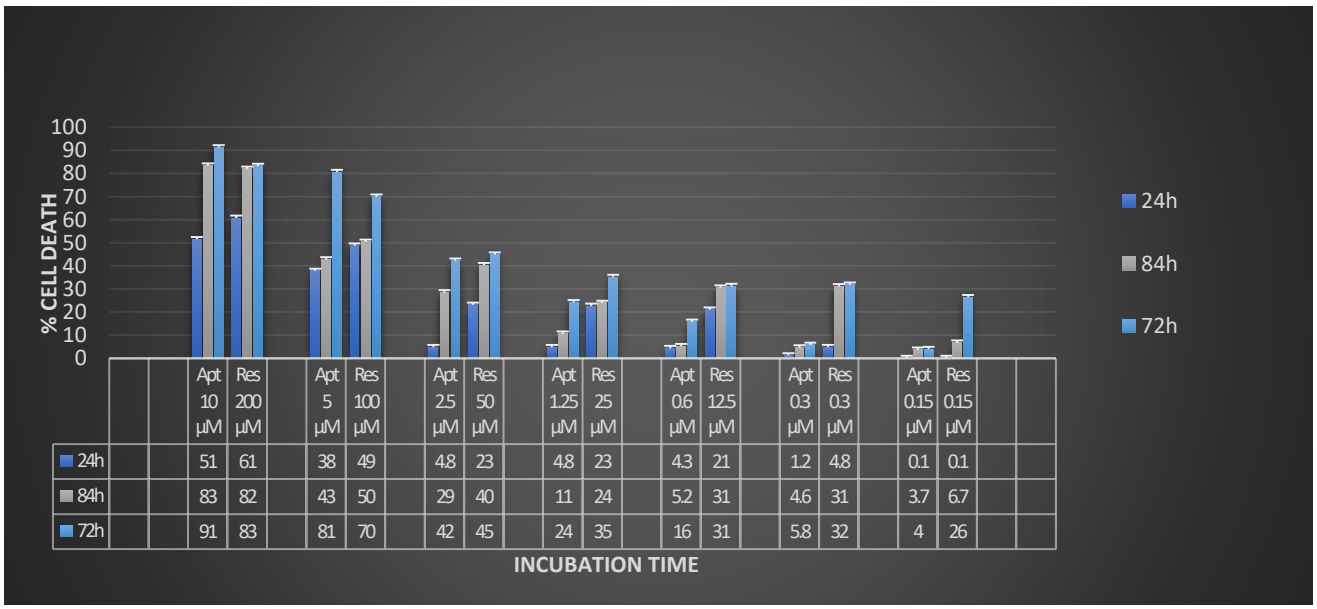

Figure 5. *In vitro* cell death percentage of the Human Acute Lymphoblastic Leukaemia Cell Line (MOTN-1) was estimated by MTT assay in 96-well plates following 24, 48 and 72 hours exposure to 0.15, 0.312, 0.625, 1.25, 2.5, 5 and 10 µM aptamer. Data is shown as % mean ± SEM of cell death for of 3 separate experiments. Treatment significantly different from the untreated controls p <0.005.

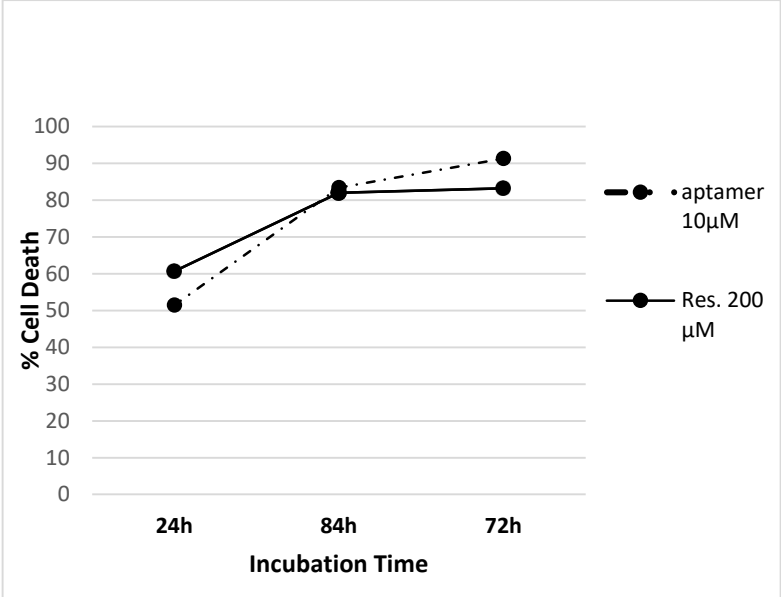

Figure 6. *In vitro* the comparison of cell death percentage of the Human Acute Lymphoblastic Leukaemia Cell Lines (MOTN-1) were treatment with 10 µM aptamer and 200 µM Resveratrol (control). The absorbance was measured at 540 nm (reference wavelength 650 nm) using a microplate reader. The results represent the mean ± SEM of 3 independent experiments. Aptamer have a strong inhibition ability for MOTN-1 cells. p <0.001 at 24, 48 and 72 hours, respectively, versus control.

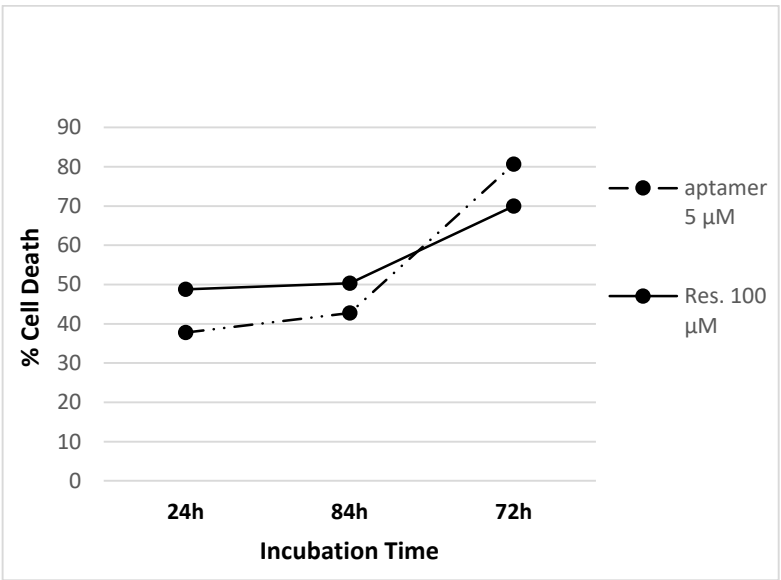

Figure 7. *In vitro* the comparison of cell death percentage of the Human Acute Lymphoblastic Leukaemia Cell Lines (MOTN-1) were treatment with 5 µM aptamer and 100 µM Resveratrol (control). The absorbance was measured at 540 nm (reference wavelength 650 nm) using a microplate reader. The results represent the mean ± SEM of 3 independent experiments. Aptamer have a strong inhibition ability for MOTN-1 cells. p < 0.005 at 24, 48 and 72 hours, respectively, versus control.

Half Maximal Inhibitory Concentration (IC_{50}) Value

The dose-response curve generated by Origin 9.1 using nonlinear regression analysis for aptamer in MOTN-1 cells are shown in Figure 8. The IC_{50} values were obtained to a range of concentrations of aptamer from 0.15, 0.312, 0.625, 1.25, 2.5, 5 and 10 µM by MTT assay. The results of IC_{50} for linear aptamer was (3.5 µM) in MOTN-1 cells.

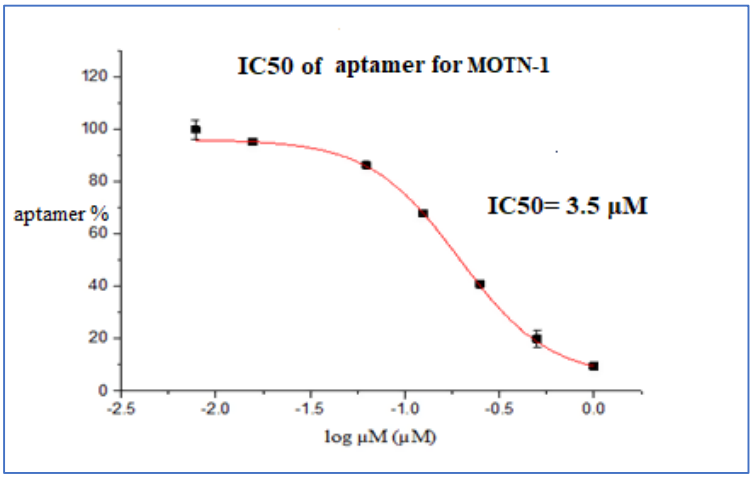

Figure 8. Dose-response curves of IC_{50} for linear aptamer: in MOTN-1 cells. It was treated for 72 hours with 0.15, 0.312, 0.625, 1.25, 2.5, 5 and 10 µM dose ranges of aptamer. The normalized dose response for aptamer was plotted over log transformed aptamer concentrations. IC_{50} values were determined using nonlinear regression analysis (Origin 9.1). Error bars represent the standard error of the mean (SEM) for triplicate data.

cDNA Microarray Analysis

An effort to comprehend differences between MOTN-1 dealt with aptamer and MOTN-1 untreated cells, gene-expression levels between these 2 were compared using a cDNA micro array.

A cDNA microarray technique was utilized in order to make comparison between the gene expression of the MOTN-1 untreated cell line and MOTN-1 treated with aptamers. The comparison of gene expression allowed us to study the differences between the said cell lines in detail. Table 1 shows the genes with the ratio of gene expression of MOTN-1 treated with aptamer and MOTN-1 untreated cells, the treated MTN-1 cells showed gene expression of more than 3 or less than 2 as compared to the untreated MOTN-1 cells. The results depicted that around 13 genes expressed differently either they were upregulated or downregulated. A total of 11 genes out of 13 differently expressed genes were upregulated. They include: dek, rac, Op18, CD6, CD58, CD106, Id2, ATF4, IRF5, ELL2, and D6 and 2 genes including GzmA, GzmK were downregulated. Molecules which are involved in hematologic disorders such as oncoproteins, chemokine receptors, surface antigens, molecules relate dto apoptosis, transcription factors and several others were encode on these genes.

Confirmation of the Gene Expression by Real-Time Quantitative RT-PCR

Although cDNA microarray was used successfully to spot the difference between the treated and untreated cell lines. The mRNA levels of selected 13 genes were observed (including 11 genes which were upregulated and 2 downregulated ones) and it was confirmed that mRNA levels also expressed differently in Atlas array. The technique used was real-time quantitative RT-PCR with SYBR GreenI. At the initial stage, the relative expression levels determined with the cDNA microarray were correlated with the LightCycler results for the all samples tested, with the result showing the dependability of our cDNA hybridization findings. In Table 1, the relative expression levels are expressed in terms of average of gene expression difference

of MOTN-1 cells treated with aptamer in comparison with MOTN-1 untreated cells in all three of the replicated experiments. The PCR amplification specificity with the LightCycler system was verified for every assay by melting curve analysis. PCR products obtained after the Gel-electrophoresis confirmed that sizes of the PCR products were similar to those of expected ones and it also indicated the specific PCR amplifications.

Table 1. Expressed genes in MOTN-1 cells compared with MOTN-1 cells treated with aptamer analyzed with cDNA array and real-time quantitative RT-PCR.

Gene Bank accession number	Gene description	cDNA array ratio	RT-PCR mean ratio
High-regulated genes			
X64229	dek (DEK oncoprotein)	4	4.6±0.4
M29870	rac (ras-like protein TC25)	2.6	2.7±0.3
J04991	Op18 (oncoprotein 18)	3.5	3.0±0.5
X60992	CD6 (CD6 antigen; TP12)	8.2	5.1±0.7
Y00636	CD58 (CD58 antigen; LFA3)	3.9	3.5±0.6
M30257	CD106 (CD106 antigen; VCAM1)	5.1	6.9±0.9
M97796	Id2 (DNA-binding protein inhibitor)	3	3.5±0.1
D90209	ATF4 (cAMP-response element binding protein)	4.3	3.6±1.3
U51127	IRF5 (interferon regulatory factor 5)	3.5	2.5±0.9
U88629	ELL2 (RNA polymerase II elongation factor)	3.2	3±0.5
Y12815	D6 (chemokine receptor D6)	3.4	3±0.2
Low-regulated genes			
M18737	GzmA (granzyme A; granzyme 1)	-2.9	-3.5±0.2
U35237	GzmK (granzyme K; granzyme 3)	-3.8	-3.7 ±0.1

Discussion

Mammalian cell has 7 SIRTs in total, SIRT1, SIRT2, SIRT3, SIRT4, SIRT5, SIRT6 and SIRT7. SIRT1 and SIRT6 are present in nucleus, SIRT7 is present in nucleolus, SIRT2 is located in cytoplasm and finally SIRT3, SIRT4, SIRT5 are present in mitochondria. SIRT1 is responsible for apoptosis, proliferation of the cells, survival of the cell, tumorigenesis, transcription silencing, the regeneration of the tissues, differentiation of the cells, producing stress response and finally regulation of the mitochondria. SIRT 2 is responsible for the regeneration of the nerves, myelination of the nerves, mitosis, aging of brain, differentiation of the adipocytes, mainataining the integrity of the genome and oxidative catabolism. SIRT3 handles the functions of the mitochondria such as TCA or tricarboxylic acid cycle, oxidation of the fatty acids, oxidative phosphorylation and the oxidative stress. SIRT4 is also responsible for the same functions in the mitochondria such as TCA cycle, fatty acid oxidation. SIRT5 aids in urea cycle. SIRT6 is responsible for two major functions i.e. silencing of the telomere phase and maintaining the stability of the genome. Finally SIRT7 is involved in rDNA transcription.

Out of these 7 SIRTs, SIRT1 has faced a lot of controversies. They have been shown to be overexpressed in many cancers. Then there are situation where they remain unchanged or reduced in different cancers. The incidence of colon cancer was shown to be decreased in one case where SIRT1 were overexpressed in the intestines. SIRT1 in this case deacetylates the oncogenes called β-catenin, which ultimately inhibits their passage and settling in the nucleus. Where any damage occurs to the DNA, aptamers travel to the site to function as genome integrity stabilizers by rapairing the damage in the DNA. Investigation of some cancers surprisingly revealed the reduced number of SIRT1 and SIRT2 in the cancer cells.(51)

These results show that SIRT1 can be used as tumour suprresor, contrastingly, other studies have revealed negative aspects of SIRT1 when it comes to treating a few cancer types. SIRT1 according to few studies have shown to be involved in inactivating the tumour suppressor p53, this inactivation is based on deacetylation of p53. Furthermore, it also inactivates the apopotis controlled by p53 especially when cell faces oxidative stress sor any damage in the DNA. Many cancers have shown overexpressed SIRT1 which leads us to the conclusion that SIRT1 might be an oncogene. This needs to be studied further as moleules like aptamers which activates the SIRT1 have shown positive results in treating cancer lines. It might lead to a gateway of new innovations and development in the modern treatment strategies of cancer.

This study is therefore, very important as it is contributing to both theoretical and practical knowledge. Theoreticaly it adds up information about how SIRT1 activators work in controlling the tumorigenesis and practically it compares the effects of aptamers, a SIRT1 activator in controlling the MOTN-1 cancer cel line.

We started with the incubation of ssDNAs with SIRT1 in a column Oligonucleotides bound with the help of buffers of increased ionic strength were separated using a high salt step. Finally, the product was amplified and clones were made using PCR technique. Ot of 72 clones obtained after the 12 rounds of selection, 50 completed sequences were used in class analysis sorted. Out of these 50 clones, 14 clones (28%) represented aptamer. Final sequence of aptamer obtaine was 5' CACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTA 3'.

Binding capacity of aptamer with the target SIRT1 is crucial. The better the binding capacity, the better would be the results. The obtained aptamer was studied for its binding capacity using SPR or Surface Plasmon Resonance technique. The aptamers were bound on a sensor chip to build a sensor surface and SIRT1 was injected. The concentrations of SIRT1 was ranging upto 800nM. About 1000-1200 RU of aptamer was adjusted on the sensor surface during the whole injecting process. The disassociation constant or kD was found out to be 48.3 ± 0.986 nM, which was low and showed higher binding affinities.

After the evaluation of the binding capacity and disassociation constant or kD of the developed aptamer, the same aptamer was used to treat MOTN-1 cell lines. MOTN-1 cells were treated with different concentrations of the aptamers i.e. 0.15, 0.312, 0.625, 1.25, 2.5, 5 and finally with 10μM. The time allowed was 24, 48, and 72 hours The maximum activity was shown by the aptamer at a concentration of about 10μM which is 51%, 83% and 91% at 24, 48 and 72 hours respectively.

On the other when the results were compared with that of resveratrol which is a natural sirtuin activator or phytoalexin (trans-3,5,4′-trihydroxystilbene). Resveratrol is obtained from

many different species of plants especially from berries, grapes and peanuts etc Plants produce resveratrol whenever they suffer a mechanical injury, excess U.V. radiation or any kind of infection such as fungal infection.(52) It was found out to be less effective even at the dose of 200µM with 61%, 82%, and 88% at 24, 48 and 72 hours respectively. However at the concentration of 5µM , the aptamer showed 38%, 43% and 81% of cell death at 24, 48 and 72 hours whereas resveratrol at around 100µM showed cell death of MOTN-1 cell line to be 49%, 50%, and 70% at 24, 48 and 72 hours respectively. When the 2.5µM aptamer and the 50µM resveratrol were used, the percentage cell death produced by aptamer was higher than that of resveratrol i.e. with aptamer 48%, 29% and 42% at 24, 48, and 72 hours respectively and the percentage cell death produced by the resveratrol at a concentration of 25µM was 23%, 24% and 35% at 24, 48, and 72 hours respectively. Furthermore, the maximum inhibitory concentration of the developed aptamer for MOTN-1 cell line was found out to be 3.5µM. Another study revealed that half maximal inhibitory concentration of circular aptamers for lung cancer, colorectal adenocarcinoma, and liver cancer was 0.32µM, 0.67µM and 0.2µM respectively. It can be concluded that aptamer can prove to be a better option when it comes to treating different cancer lines. However, to further authenticate our results we compared both our treated an untreated cell lines for the gene expression. The technique used was cDNA microarray analysis and it showed that 13 genes were expressed differently in treated and untreated MOTN-1 cells. Finally, the RT-PCR technique was used to counter check the results obtained from the cDNA microarray analysis. Bothe showed the same results with 11 genes being upregulated and two being downregulated.

When compared with other sirtuin activators such as resveratrol have very limited bioavailability which makes it less suitable to be taken orally. Moreover results obtained from rodents and those obtained from humans are also inconsistent. Even the change of route of administration, specie, or dose etc. can lead to different results in case os resveratrol. Therefore when it comes to treating the cancers in human the use of resveratrol supplementation needs to be studied further.

The novelty of this study resonates the emerging aptamer related technologies and their use as targeting ligands especially for the oncogenes. Aptamers are oligonucleotides with different structure of RNA and DNA. They have high binding affinity as discusse above and high efficacy too when compared with natural sirtuin activators such as resveratrol. Their interaction with other target molecules depends on their tertiary structures with complex folding. Even with a highly complex folded structure they have low molecular weights and mimic small protein molecules. When compared to antibodies which are conventionally used in the treatment of different cancer types, they are easy to modify and can be synthesized in vitro. Being easy to modify they an easily conjugate with chemicals used for diagnostic and therapeutic purposes such as nanoparticles, chemotherapeutic agents and more.(53) Above that, aptamers can also be stored for a longer duration as compared to antibodies and have very low immunogenicity too. The results shown in this study and those obtained from published literature gives a clear picture of the future of aptamers for the treatment of leukemic cancer in the coming future.

This study also urges the researchers to address the challenges of using the aptamers. The major disadvantages are shorter half-lives and quick renal excretion. These problems, however, can be addressed by making chemical modifications to the structure of developed aptamers or modification the linear aptamer to circular aptamer where it is very stable .

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