Original Article

# Drug Discovery And Antiproliferative Effect Of Linear And Circular Aptamers On Colon Cancer

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## Abstract

The word 'Sirtuin' or Sir2 proteins are a class of proteins that possess either mono-ADP-ribosyltransferase, or deacylase activity, including deacetylase. SIRT1 is the most studied mammalian Sirtuins and predominantly localised in the nucleus and cytoplasm. Many Sirtuins targets are involved in cancer and in many types of cancers, SIRT1 is found to be overexpressed. Recent observations support SIRT1 being both an oncogene and a tumour suppressor, depending on the cancer etiology and type of tissue. To answer the question "How can SIRT1 activator (BAS Aptamers) behave as a tumor suppressor in colon cancer?" In this regard, we propose to study the characterisation of the interactions between BAS Aptamers (linear3, linear4, circular3 and circular4) and SIRT1 in human colon cancer cells (HCT116) as the first step towards the development of an alternative chemotherapy for colon cancer diseases. The objectives of current study are to investigate the effects of selected BAS aptamers (Linear3, Linear4, Circular3, and Circular4) on colon cancer cell line. In conclusion, a pharmacological activation of SIRT1 enhanced cell death suggesting a tumour suppressive function of SIRT1 and the high-affinity SIRT1-aptamers identified in this study may be used in the future for cancer treatment.

**Keyword:** SIRT1, HCT116 cell line, IC50.

**Introduction:** SIRT1 has emerged as a drug development target for treating age-dependent diseases such as cancer (1). An excessive amount of SIRT1 is expressed by primary cells, and these levels usually fall during cellular aging so that replicative senescence is avoided and one is secured from tumourigenesis (2). Replicative senescence is a kind of tumour suppression. SIRT1 is crucial for restricting replicative lifespan which suggests that SIRT1 works as a tumour suppressor. Mouse embryonic fibroblasts (MEFs) derived from SIRT1-null mice are prone to spontaneous immortalisation, suggesting that SIRT1 behaves as a growth-suppressive gene in culture (2). Furthermore, hematopoietic stem cells from SIRT1-null mice have increased proliferation potential, and shRNA knockdown of SIRT1 in human fibroblasts accelerates cell proliferation. SIRT1 has also been shown to inhibit androgen receptor-dependent cell proliferation in prostate tumour cells (3). Recent publications also showed that transgenic overexpression of SIRT1 in the intestine inhibited polyp formation in the $Apc^{Min}$ mice, (4), whereas SIRT1 deficiency led to increased tumour formation in p53-null mice (5). These observations suggest that SIRT1 may suppress tumour growth under certain conditions, and that SIRT1 activators could be used for cancer treatment or prevention (6). The SIRT1 activator may work as a beneficial chemopreventative agent in case SIRT1 behaves as a tumour suppressor (7). Activation of SIRT1 will: (i) increase expression of PGC-1α with reduction of reactive oxygen species (ROS), (ii) interaction with FOXO3 for antioxidants effect and

arrest of cell cycle, (iii) interaction with NF-κB and Ku70 reducing inflammation and leading to apoptosis, (iv) epigenetic modifications by modifying the expression of H3-TriMeK9, H4-MeK20, Ac-H4-K16, Ac-H3-K9, Ac-H1-K26, H3-MeK79 and (vii) in neurogenesis increasing the expression of deacetylation RARβ, reducing the β-amyloid plaques and increasing deacetylation of tau protein (T protein that stabilise microtubules), reducing the tangles. In addition, a recently identified endogenous activator of SIRT1 designated active regulator of SIRT1 (AROS) binds to the N-terminus of SIRT1 and potentiates its deacetylase activity toward p53 in the damage response (8) as shown in figure 1.

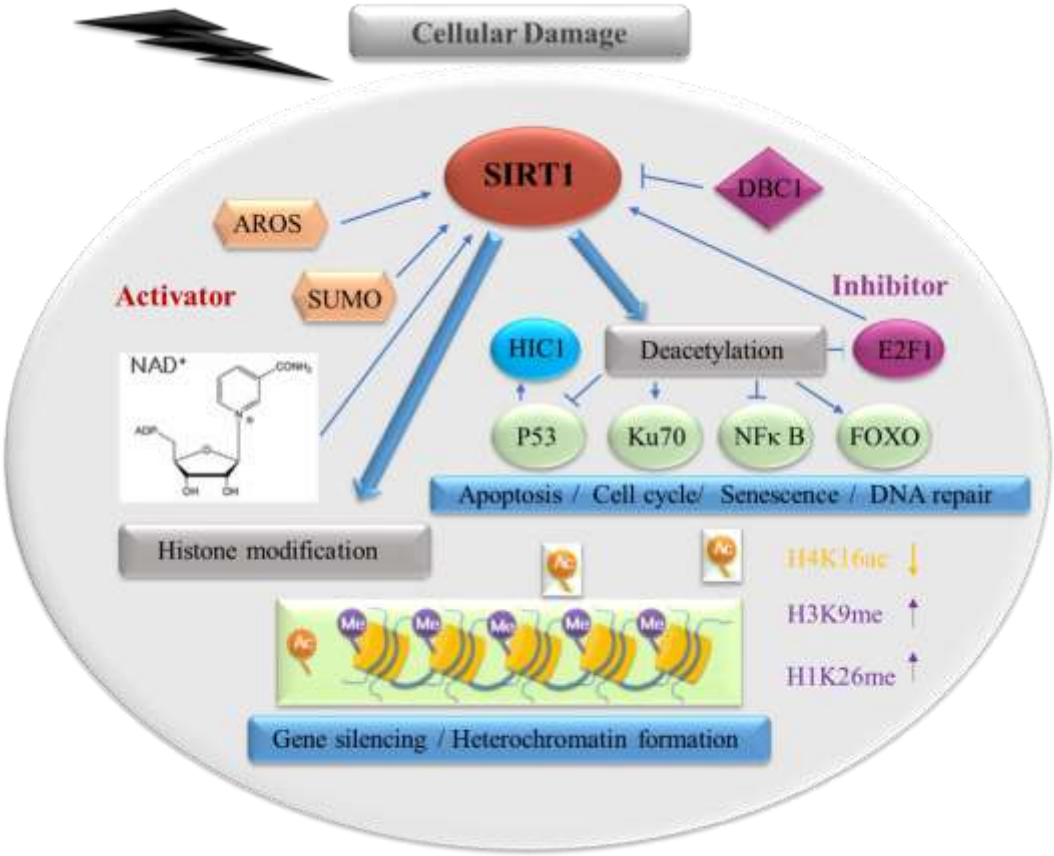


**Figure 1:** SIRT1's anticancer activity (1).

Studies have shown that the SIRT1 activator resveratrol, a polyphenol found in wines and thought to harbor major health benefits, induces apoptosis in response to Tumour Necrosis Factor (TNFα) via NF-κB inhibition, and has chemopreventive activity against various cancers, including leukemia, skin cancer, and prostate cancer (9; 10; 11). Resveratrol also induces autophagy, and it has been shown that this occurs in a SIRT1-dependent manner (12). On the other hand, in a cell-culture model of rotenone-induced cell death, resveratrol was reported to protect against rotenone-induced apoptosis and enhance degradation of α-synucleins (a protein that is abundant in the human brain), which was shown to occur by induction of autophagy (13). The resveratrol derivative Longevinex (a product that activates 9 times more genes than plain resveratrol) has the curious effect of increasing autophagy after prolonged administration, and this correlates with increased SIRT1 levels, as well as FOXO nuclear translocation (14). Subsequent studies in this field are going to make evident the exact function of SIRT1 at the colon cancer site and it is hoped that new chemotherapeutic functions of SIRT1 activators are going to be determined. In accordance with this, it is suggested that very selective ligands such as aptamers, should be created and investigated in colon cancer cell lines for the regulated activity of SIRT1 as shown in figure 2. The purpose of which would be to find out the manner in which SIRT1 acts as a tumour suppressor. Aptamer is single-stranded DNA or RNA (ssDNA or ssRNA) molecules that can bind to pre-selected targets including proteins and peptides with high affinity and specificity are termed as aptamers (15; 16). Their 3D structure enables them to bind with a specific target molecule with considerable high specificity and

affinity (17). The 3-D conformations of oligonucleotides, rather than the nucleotide sequence are the responsible for binding potential of aptamers (18).

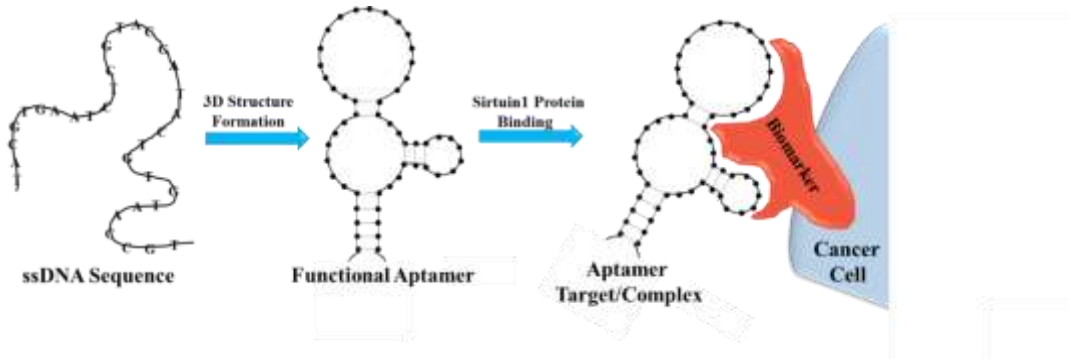


**Figure 2:** Diagram of aptamer binding to SIRT1 protein (19).

## Methods:

**Aptamers preparation**: The aptamers used for this study was made of a 40-77nt. The aptamers were referred to as the single stranded DNA with the nucleotide sequence as shown in table 1 below. The aptamer was desalted and lyophilized by the manufacture (BioNeer, Korea).

| DNA oligoes | Sequence (5`-3`) | Supplier |
|---|---|---|
| **-L3 aptamer (Linear3)** | CACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTG TTTA | Alpha DNA, Canada |
| **-L4 aptamer (Linear4)** | TTCGGAAGAGATGGCGAC CACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTG TTTA CGAGCTGATCCTGATGGAA | Alpha DNA, Canada |
| **-C3 aptamer (Circular3)** | CGAGTGGGTTACATCGAAACTGGATCTCAACAGCG GTAAC | Alpha DNA, Canada |
| **-C4 aptamer (Circular4)** | TTCGGAAGAGATGGCGAC CGAGTGGGTTACATCGAAACTGGATCTCAACAGCG GTAAC CGAGCTGATCCTGATGGAA | Alpha DNA, Canada |

**-Cell Culture:** Human colorectal carcinoma cells (HCT116) were originally obtained from Sigma Aldrich. HCT116 cells were maintained in McCoy's 5A medium (Gibco) supplemented with 10% fetal bovine serum FBS and 1% L-Glutamine as well as to 1% Penicillin-Streptomycin-Amphotericin B 100X as antiseptic (Invitrogen). Cells were cultured in 75 $cm^2$ flasks and incubated in 5% $CO_2$/ 95% humidified air at 37° C. Once the cells reached 90% confluence, flasks containing HCT116 cells were passaged under sterile conditions. The cells were washed with 5 ml of phosphate buffered saline solution (PBS) and then incubated for 2 min in trypsin solution at 37° C to allow cells to detach from the bottom of the flask. An equal volume of complete growth media was added and the cell suspension was transferred into a 50 ml conical tube. Cells were then centrifuged at 1200 rpm for 3min. The supernatant was discarded and the cell pellet resuspended in fresh supplemented growth media. Cells were then counted under the microscope on a haemocytometer and used as required.Following trypsinisation of a confluent 75 $cm^2$ flask, the cell suspension was centrifuged at 1200 rpm for 3 min. The cell pellet was then resuspended in 4ml freezing medium and 1ml aliquots were added to cryovials. The cells were stored at -80° C for 24h and were stored under liquid nitrogen for long-term storage. Cells stored under liquid nitrogen were quickly thawed at 37° C and added to 10 ml fresh growth media. The cells were harvested by centrifugation and resuspended in 25 ml of fresh medium and transferred to a 75 $cm^2$ flask and grown (20,21).

**-Cell Viability:** The MTT assay was used to assess the effects of aptamer on colon cancer cell viability. A 100 µl from all cells suspensions (HCT116) were dispensed into 96-well flat-bottom tissue culture plates at concentrations of 5 x $10^3$ cells per well and incubated 24h under standard conditions; 4 x $10^3$ cells/well for 48h incubation, and 3 x $10^3$ cells/well for 72h incubation. After 24h, the cells were treated with 2.5 µM aptamers (linear3, linear4, circular3 and circular4) then cells were exposed to 50 µM TBHP (Tert-Butyl Hydrogen Peroxide) before and after 4h from adding the aptamers as shown in figure 3. After a recovery period 24h,48h and72h, 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 4h at 37° C. After 4h 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 multiscan reader at a wavelength of 540 nm and correcting for background absorbance using a wavelength of 650 nm (22).

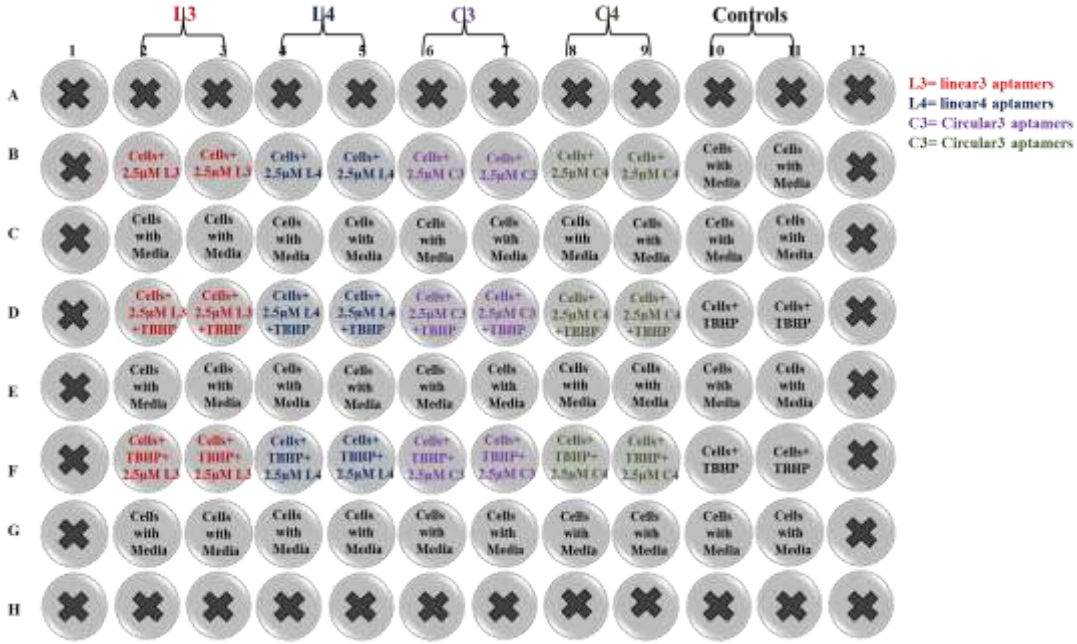


**Figure 3:** 96-well plate template for MTT assay. Column 2 and 3 are duplicate of linear3 aptamers, whereas line 2B and 3B contains cells and 2.5 µM L3 aptamer; line 2D and 3D contains cells and 2. 5µM L3 aptamer then after 4h added 50 µM TBHP; line 2F and 3F contains cells and 50 µM TBHP then after

4h added 2.5 µM L3 aptamer. Column 4 and 5 are duplicate of linear4 aptamers, whereas line 4B and 5B contains cells and 2.5 µM L4 aptamer; line 4D and 5D contains cells and 2.5 µM L4 aptamer then after 4h added 50 µM TBHP; line 4F and 5F contains cells and 50 µM TBHP then after 4h added 50 µM L4 aptamer. Column 6 and 7 are duplicate of circular3 aptamers, whereas line 6B and 7B contains cells and 50 µM C3 aptamer; line 6D and 7D contains cells and 2.5 µM C3 aptamer then after 4h added 50 µM TBHP; line 6F and 7F contains cells and 50 µM TBHP then after 4h added 2.5 µM C3 aptamer. Column 8 and 9 are duplicate of circular4 aptamers, whereas line 8B and 9B contains cells and 2.5 µM C4 aptamer; line 8D and 9D contains cells and 2.5 µM C4 aptamer then after 4h added 50 µM TBHP; line 8F and 9F contains cells and 50 µM TBHP then after 4h added 2.5 µM C4 aptamer. Column 10 and 11 are duplicate of controls, whereas line 11B, 12B,1-12C1-12E and 1-12G contains cells with media; line 10D, 11D, 10F and 11F contains cells with TBHP.

**-Determination the Half Maximal Inhibitory Concentration (IC50) Value:** The $IC_{50}$ of the drug can be determined by constructing a dose-response curve and examining the effect of different concentrations of the antagonist on reversing agonist activity. $IC_{50}$ values can be calculated for a given antagonist by determining the concentration needed to inhibit half of the maximum biological response of the agonist. $IC_{50}$ values are very dependent on conditions under which they are measured. In general, the higher concentration of inhibitor, the more agonist activity will be lowered. $IC_{50}$ value increases as agonist concentration increases. Furthermore, depending on the type of inhibition other factors may influence $IC_{50}$ value. According to the in vitro MTT assay, the $IC_{50}$ represents the concentration of the tested C3 aptamer that is required for 50% inhibition of the cell viability. Based on the obtained data using the in vitro MTT assay, the $IC_{50}$ values for C3 aptamer at 72h after the cells exposure to C3 aptamer. To determine the $IC_{50}$ values, the concentration range used of C3 aptamer was 0.0078 - 1.00 µM (23).

**-Data Analysis:** All statistical analysis of MTT assay and $IC_{50}$ data of SIRT1 aptamers (linear3, linear4, circular3 and circular4) on HCT116 cells were performed using the nonlinear curve fitting software prism 8.1 software. Comparison between all groups within the same plate of MTT were evaluated by one-way ANOVA with, comparison between the same group within the same plate of MTT assay were evaluated by paired t-test using (IBM SPSS Statistics 20) statistical software. Values of $p < 0.05$ were considered statistically significant.

**Results:** The results of all the experiments in this study are representing the results of three independent experiments. The concentration of aptamers in cell viability experiments are 2.5 µM because this concentration is the best one after doing the optimisation experiments to choose the best concentration between 1-4 µM of C3, C4, L3 and L4 aptamers. HCT116 cells were exposed to 2.5 µM L3, L4, C3 and C4 aptamers at 24, 48 and 72h to estimate the effect of aptamers on HCT116 cell viability. L3, C3 and C4 aptamers significantly reduced cell viability at 2.5 µM, $p < 0.05$, 0.005 and 0.001 at 24, 48 and 72h respectively as shown in figures 4, while L4 aptamer was not effected on HCT116 cell viability at the same concentration ($p > 0.05$).

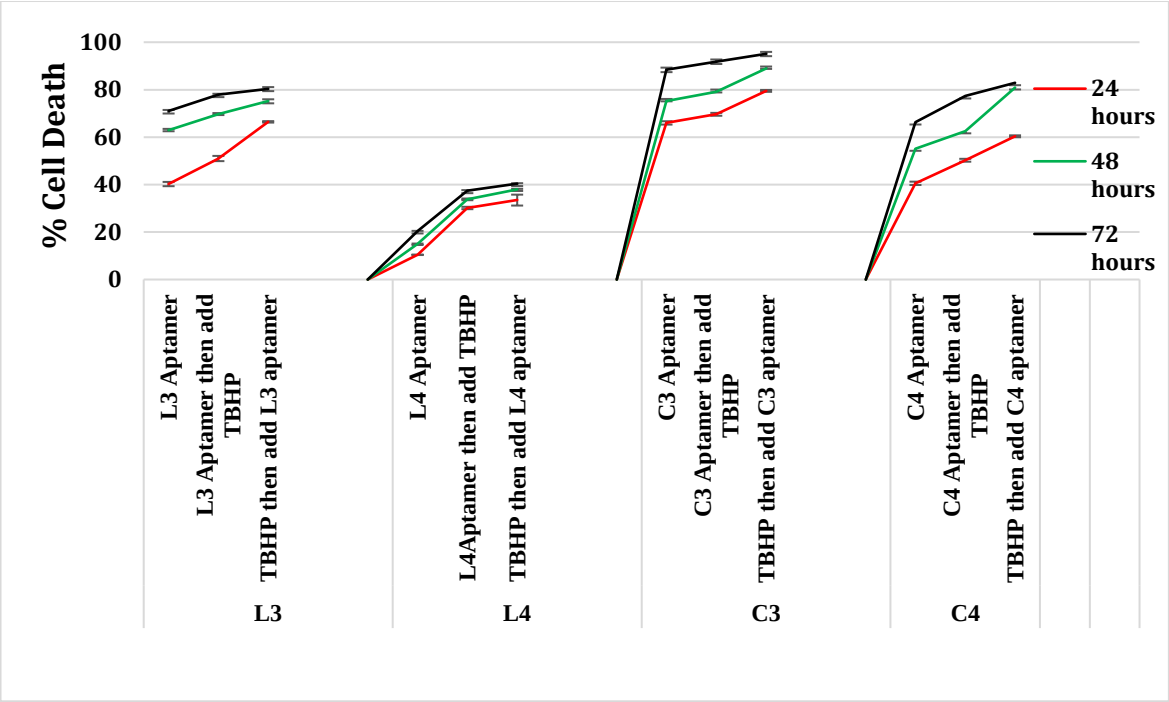


**Figure 4:** In vitro cell death percentage of the colorectal adenocarcinoma (HCT116) cells was estimated by MTT assay in 96-well plates following 24, 48 and 72h exposure to 2.5 µM (L3, L4, C3 and C4) aptamers and 50 µM TBHP. Data is shown as % mean ± SEM of cell death for 3 separate experiments. Treatment significantly different from the untreated controls $p < 0.005$.

HCT116 cells which were treated with 50 µM TBHP also showed a reduction of 30% in cell viability after 24h compared to untreated cells; however, the difference was not statistically significant. The reduction in cell viability became more pronounced after treated with 2.5 µM L3, C3 and C4 aptamers and 50 µM TBHP, $p < 0.005$ (figure 4). In addition, L4 aptamer was not effected to HCT116 cells viability after treated with 50 µM TBHP, $p > 0.05$. Based on the results presented in figure 4, cells exposed to 2.5 µM L3, C3 and C4 aptamers after 24h were presented a statistically significant increase of cell death when compared to untreated cells ($p < 0.005$). 2.5 µM aptamers and 50 µM TBHP treatment after 72h caused a cell death increase of 80%, 95% and 82% for L3, C3, and C4 respectively. Between the groups of aptamers, C3 aptamer was showed significantly higher percentage of cell death than L3, L4 and C4 aptamers ($p < 0.005$). The dose-response curve generated by prism pad 8 using nonlinear regression analysis for C3 aptamer in HCT116 cells are shown in figure 5. The $IC_{50}$ values were obtained to a range of concentrations of C3 aptamer from 0.0078 – 1 µM by MTT assay. The results of $IC_{50}$ for C3 aptamer was 0.38µM in HCT116 cells.

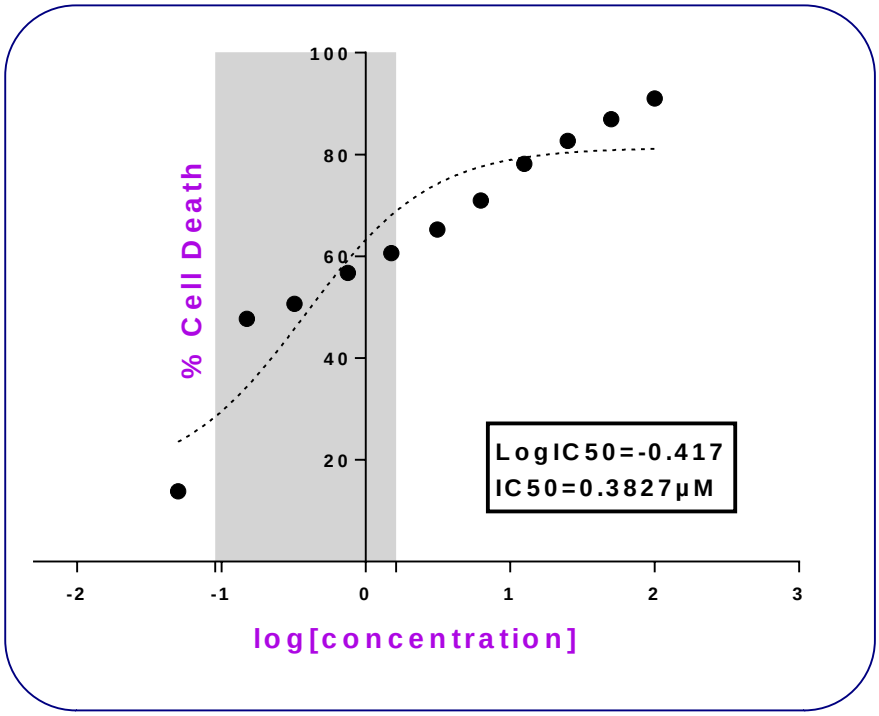


**Figure 5:** Dose-response curves of $IC_{50}$ for C3 aptamer. HCT116cells were treated for 72h with 0.0078, 0.0156, 0.0312, 0.625, 0.125, 0.25, 0.5 and 1 µM dose ranges of C3 aptamer. The normalised dose response for C3 aptamer was plotted over log transformed aptamer concentrations. $IC_{50}$ values were determined using nonlinear regression analysis (Origin 9.1). Error bars represent the standard error of the mean (SEM) for triplicate data.

**Discussion:** This study has investigated oligonucleotide aptamer as can use for anticancer drug discovery. In doing so, the work in this project has provided information in greater detail regarding the use of MTT assay to investigate the inhibitory effect of SIRT1-aptamers on viability of cancer cells (HCT116). SIRT1 is implicated in cell proliferation (24). Most studies have established that SIRT1 was involved in cancer cells. For instance, Li et al.(25) were established that downregulation of SIRT1 inhibits proliferation of breast cancer cells (BT-474, MDA-MB-231, MDA-MB-435, SK-BR-3), and inhibition of SIRT1 blocks proliferation of chronic lymphocytic leukemia cells. In contrary to this, increased expression of SIRT1 after resveratrol treatment inhibits proliferation of osteosarcoma cells (HOS, Saos-2, U2OS and MG-63) (26), breast cancer cells (MCF-7, MDA-MB-231) (27) and human colon cancer cells (Caco-2) (28). In the current study, our experiments using cultured cancer cell line (HCT116) demonstrates that SIRT1 activated by L3, L4, C3 and C4 aptamers has properties of a growth suppressor. Kabra (29) was found that knockdown of SIRT1 increases the rate of tumour growth by enhancing cell proliferation, whereas overexpression of SIRT1 reduces tumour initiation and growth in nude mice, this results is agreement with our suggestion that the pharmacological activation of SIRT1 by aptamers decreases the rate of cell viability in cancer cell line (HCT116) as shown in figure 1. Together, these results suggest that SIRT1 functions as a context-dependent tumour suppressor where activation of SIRT1 suppress tumour initiation and promote cell death. SIRT1 has a well-established anti-apoptotic function which has led to the presumption that it acts as an oncogene. However, a study showed that transgenic mice overexpressing SIRT1 reduced the development of neoplasia in the intestine caused by $Apc^{Min}$ mutation suggesting a tumour suppressive role of SIRT1 (30). Wang et al., (31) demonstrated that Sirt1+/- mice showed increased tumour incidence when crossed to a p53+/- background. These genetic models strongly suggest that SIRT1 has properties of a tumour suppressor and our results lend further support to this function of SIRT1. It is noticeable that our results regarding

the anti-proliferative properties of SIRT1 contradict many published studies that show an anti-apoptotic function of SIRT1. According to these published studies, tumour cells undergo apoptosis or growth arrest after transient knockdown of SIRT1 or treatment with SIRT1 inhibitors such as sirtinol, splitomicin and cambinol (32, 33, 34). Based on our results, aptamers have increased the activity of SIRT1 in cancer cell lines thereby sensitising these cells to DNA damage-induced apoptosis and promotes cell death. A possible explanation for the discrepancies between the studies might be that, in the studies published by other research groups tumour cells treated with SIRT1 shRNA (small hairpin RNA, is an artificial RNA molecule with a tight hairpin turn that can be used to silence target gene expression via RNA interference) may be sensitised to apoptosis due to additional transfection associated stress. Furthermore, the off-target toxicity of the first-generation small molecule SIRT1 inhibitors could be responsible for the cell death or growth arrest responses that they observed (29). In fact, the recent development of the nanomolar SIRT1 inhibitor EX-527 demonstrated that specific inhibition of SIRT1 alone does not cause apoptosis in tumour cell lines (35; 29). Several studies have suggested that SIRT1 may act as an oncogene based on the correlation of higher than normal expression levels of SIRT1 in certain tumours compared to normal tissue (36; 37; 38). In contrast, Kabra, 29) established that SIRT1 levels are variable in different stages of colon cancer tumours, such a staining pattern can be interpreted as SIRT1 having both oncogenic and tumour-suppressive properties which are consistent with the pleiotropic effects of SIRT1, i.e. anti-apoptotic and growth suppressive depending on cellular context and a subset of tumours down regulate SIRT1 to obtain a proliferation advantage, whereas some could increase SIRT1 expression to benefit from its anti-apoptotic function. Although the mechanism by which SIRT1 inhibits cell proliferation remains to be further investigated, previous studies suggested that inhibition of E2F1 is partly responsible for this observed effect. SIRT1 interacts with E2F1, inhibits E2F1 acetylation, and is recruited by E2F1 to target promoters (39). When expressed at high levels, SIRT1 is a potent inducer of G1 arrest (29). Indeed, our results confirmed these previous suggestions that activators of SIRT1 by aptamers could have therapeutic potential as an anti-cancer target. The mechanism underlying the expression control of SIRT1 is poorly understood. To the best of our knowledge, we demonstrate here for the first time that aptamers induce the expression of SIRT1.The results observed in this study are consistent with several research groups have reported that resveratrol which activator SIRT1 was inhibited much more cancer cells proliferation such as Caco-2 (28), MCF-7, A549 (27), U2OS (26), MDA-MB-468 (40) and HepG2 (41; 42). The $IC_{50}$ of C3 aptamer was very low concentration (0.3 µM) in HCT116 ells as shown in figure 5. This low concentration of $IC_{50}$ is very good results because the low dose of C3 aptamer can inhibit the growth of cancer cells. In summary, our study provides important information regarding SIRT1 aptamers effect on colon cancer cells. The results presented here suggest that C3 aptamer might be useful in the treatment of this type of cancer more than the other types ( L3, L4, C4) aptamers because its properties of a growth suppressor for specific killing of the tumour cells only avoiding unpleasant side effects from damage to the rest of the body. More studies are needed, for example, the cellular mechanism of action of SIRT1 in aptamer-mediated apoptosis demands further investigation and, furthermore, the C3 aptamer-induced apoptosis of these cells needs to be investigated in appropriate in vivo models.

**Conclusion:** Using MTT assay we have produced an aptamers which is i) enters the cells and ii) interacts only with SIRT1, iii) active on colon cancer cells, and iv) the mode of action is through activation of SIRT1.

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