

Anticancer and apoptotic activities of SIRT1 activators through cell cycle arrest in Capan-2 human pancreatic carcinoma cell lines

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Abstract

A lot of novel SIRT1 activators were synthesized and estimated for their antitumor activities on pancreatic cancer cell lines, apoptosis induction, and cell cycle effects, but they contain side effects, non-direct binding to SIRT1 and high toxicity. There is, therefore, a need to develop selective and relatively non-toxic treatment regimens directed to activate SIRT1 at reducing the morbidity and mortality associated with pancreatic cancer. SIRT1 aptamer was originally designed for use in cancer treatment. However, the anti-cancer effects and the action mechanisms of SIRT1 aptamer remain elusive. In current study, the effects of SIRT1 aptamer at different concentration on the growth of human pancreatic cancer cells (Capan-2) were evaluated by study the cell apoptosis and cell cycle arrest. Taken together, the results indicated that SIRT1 aptamer induced apoptosis and cause cell cycle arrest in the S phase and could be a promising source for developing natural therapeutics for pancreatic cancer.

Keywords: cell cycle arrest, apoptosis, SIRT1, flow cytometry

INTRODUCTION

According to the recent advances in chemotherapy, pancreatic cancer remains a deadly disease and is the third leading cause of cancer related death in the United States [1]. Therefore, new therapeutic options are highly needed. Drugs for the pancreatic cancer are generally focused on preventing of molecular signaling pathways, cell cycle arrest, and incitement of apoptosis. To design novel therapies for pancreatic cancer treatment subsequent studies in this field are going to make evident the exact function of SIRT1 at the pancreatic cancer site. They hoped that new bio therapeutic functions of SIRT1 activators are going to be determined. SIRT1 aptamer was originally designed for use in cancer treatment [2]. Because of their high binding affinity and specificity, aptamers become an essential target for cancer drug development [3]. Indeed, it is logical to speculate that that very selective ligand SIRT1 aptamer can be possessed anti-cancer activity includes pancreatic cancer [4, 5, 6]. Therefore, the mechanism of effect the SIRT1 aptamer on pancreatic cancer blocking by promoting the growth-inhibitory in pancreatic cancer cell line by revealed the decrease level of intracellular ROS production. Through direct deacetylation the inactivation of the p65 subunit of NF- κ B is caused by SIRT1. Inhibition of NF- κ B causes suppression of the iNOS (inducible nitric oxide synthase are a family of enzymes catalyzing the production of nitric oxide (NO) from L-arginine) and eventually could decrease the cellular ROS load [7]. Cho *et al*, were found that activation of SIRT1 by resveratrol, activator of SIRT1, can be caused the down-regulated cyclin-D1, which is a target element of β -catenin and inhibited the Panc-PAUF cancer cells proliferation. Thence, it can be concluded that the activation

of SIRT1 may be a therapeutic approach in treating pancreatic cancer cells [8]. Accordingly, in the present study, SIRT1 aptamer was tested its antitumor activity *in vitro* using cell cycle arrest and induction of apoptosis in the human Capan-2 pancreatic cancer cell line. Furthermore, the results suggested that SIRT1 aptamer treatment significantly induced apoptosis and cause cell cycle arrest in the S phase and these findings suggested that SIRT1 aptamer could be a potential agent for the treatment of pancreatic cancer.

METHODS

Cell culture

Cell lines of human pancreatic cancer (Capan-2) was provided from American Type Culture Collection ATCC, USA. McCoy's 5A Medium was used to preserve Capan-2 cell line. These media were enhanced with 1% L Glutamine and 10% fetal bovine serum FBS as well as to 1% Penicillin-Streptomycin-Amphotericin B 100X as antiseptic. Cell lines were cultured in 75cm² flasks and incubation carried out at 37°C in 5% CO₂/95% humidified air. Once the cell lines reached 90% confluency, flasks containing Capan-2 was kept under sterile conditions. Five ml of phosphate buffered saline were added to wash the cell lines. PBS is often used in cell biology experiments to maintain the osmolarity of the cells. It contains salt ions, which balances the amount of salt ions inside the cell then the cell line incubated for 2 min with 1 ml trypsin solution at 37°C in 5% CO₂/95% humidified air, to separate the cell lines from the flask bottom. Five ml of complete growth media was added and the suspension of cell line was moved into a 50ml conical tube. Centrifugation of the cell line was performed at 1200 rpm for 3 min. Supernatant was separated and the cell pellet underwent

Anticancer and apoptotic activities of SIRT1 activators through cell cycle arrest in Capan-2 human pancreatic carcinoma cell lines

re-suspended in fresh supplemented growth media. Cell lines were counted under the microscope on a haemocytometer and used as required. The cell lines were stored for 24hrs at -80 °C and moved to liquid nitrogen. Afterwards, the cell lines were rapidly dissolved at 37 °C and then 10 mL of fresh growth media was added and put in a 75 cm² flask and grown as earlier explained [9].

Apoptosis analysis

2.5×10⁵ of Capan-2 cells were seeded in 6-well plates in triplicate. After being treated the cells with different concentrations of SIRT1 aptamer (0.25, 0.5, 1, 1.5 and 2 μM) for 72 h, the cells were harvested by trypsinization, washed twice with 500 μl cold PBS and re-suspended in binding buffer. After that, the cells stained with 5 μl of Annexin V-FITC (Elabscience, USA) and 5 μl propidium iodide PI (is a common DNA dye that is not permeable to cell membrane), then incubated for 15 min in the dark. After 15 min the samples with Annexin V-FITC binding were analyzed by flow cytometry (Ex = 488 nm; Em = 530 nm) using FITC signal detector (usually FL1) and PI staining by the phycoerythrin emission signal detector (usually FL2) (FACSCalibur flow cytometer, USA). The percentage of specific apoptosis was calculated by subtracting the percentage of spontaneous apoptosis of the relevant controls from the total percentage of apoptosis [10].

Cell cycle analysis

The cell cycle analysis was determined by seeded the 2.5×10⁵ of Capan-2 cells were seeded in 6-well plates in triplicate. After being treated with different concentrations of SIRT1 aptamer (0.5 and 1 μM) for 72 h, the cells were harvested by trypsinization and fixed with 70% (v/v) ice-cold ethanol at 4°C overnight. After washing with ice-cold PBS,

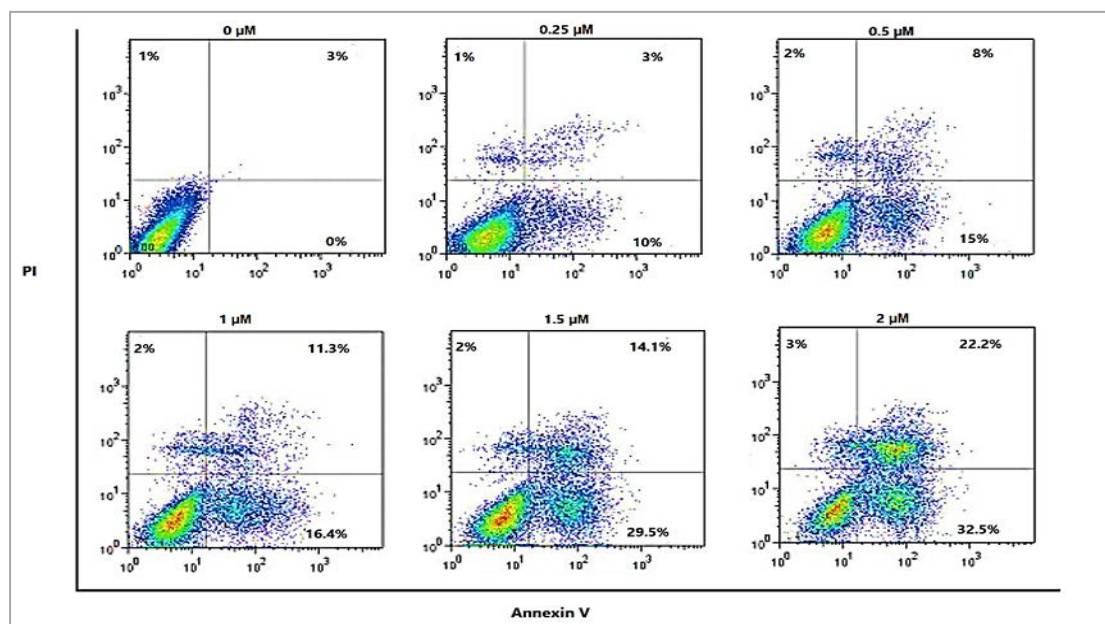
staining solution containing 0.2% NP-40, RNase A (30 μg mL⁻¹) and propidium iodide (PI, 50 μg mL⁻¹) in a phosphate-citrate buffer (pH 7.2). Cellular DNA content was analyzed by flow cytometry (FACSCalibur flow cytometer, USA). The results were displayed as histograms [11].

Statistical analysis

All statistical analyses were performed via one-way ANOVA using SPSS for Windows. Each experiment was replicated at least three times. The values were indicated as mean ± SD, p < 0.05.

RESULTS

In order to further confirm whether that SIRT1 aptamer induces apoptosis in pancreatic cancer cell lines (Capan-2), a flow cytometry study with Annexin V-FITC/PI double staining was implemented on SIRT1 aptamer against the Capan-2 cell line because SIRT1 aptamer exhibited excellent cytotoxic activities against all pancreatic cancer cell lines tested [12] (AsPC-1, Capan-2, and BxPC-3: data not shown). In view of the mentioned growth-inhibitory effects of SIRT1 aptamer against pancreatic cancer cell lines, we were interested in determining whether SIRT1 aptamer also induced apoptosis in Capan-2 cell lines. As seen in Fig. 1, after treatment of Capan-2 cell lines with SIRT1 aptamer at 0.25, 0.5, 1, 1.5 and 2 μM for 72h, the apoptosis rates were 13.00 ± 0.48%, 23.00 ± 1.55%, 29.70 ± 2.13%, 43.6 ± 3.16% and 54.70 ± 2.37%, respectively. These data were obviously higher than that for the blank control group (3.00 ± 0.09%, DMSO) and the difference was statistically significant (p < 0.05) when the concentration of SIRT1 aptamer reached 1.5 and 2 μM. These results indicated that SIRT1 aptamer could induce apoptosis of Capan-2 cell lines in a dose dependent manner. Data for the apoptosis induction in Capan-2 cell



the fixed cells were incubated for 30 min in the dark with a

lines are summarized in Table 1.

Figure 1. Apoptosis induction. Capan-2 cell lines were treated with 0.3% DMSO and SIRT1 aptamer at 0.25, 0.5, 1, 1.5 and 2 μM for 72h. Cells were double-stained with AnnexinV-FITC (green) and PI (red), analyzed for apoptosis by DNA flow cytometry. The data indicate the percentage of intact cells (AV-/PI-) and different stages apoptotic cells (AV+/PI-, AV+/PI+ and AV-/PI+) are presented.

All experiments were performed in triplicate and gave similar results.

Table 1. Apoptosis induction in human pancreatic cancer cell line (Capan-2) after treatment for 72 h with DMSO and SIRT1 aptamer at 0.25, 0.5, 1, 1.5 and 2 μM.

Conc. μM	DMSO	SIRT1 aptamer
0.25	3.00 ± 0.09	13.00 ± 0.48
0.5	3.00 ± 0.09	23.00 ± 1.55
1	3.00 ± 0.09	29.70 ± 2.13

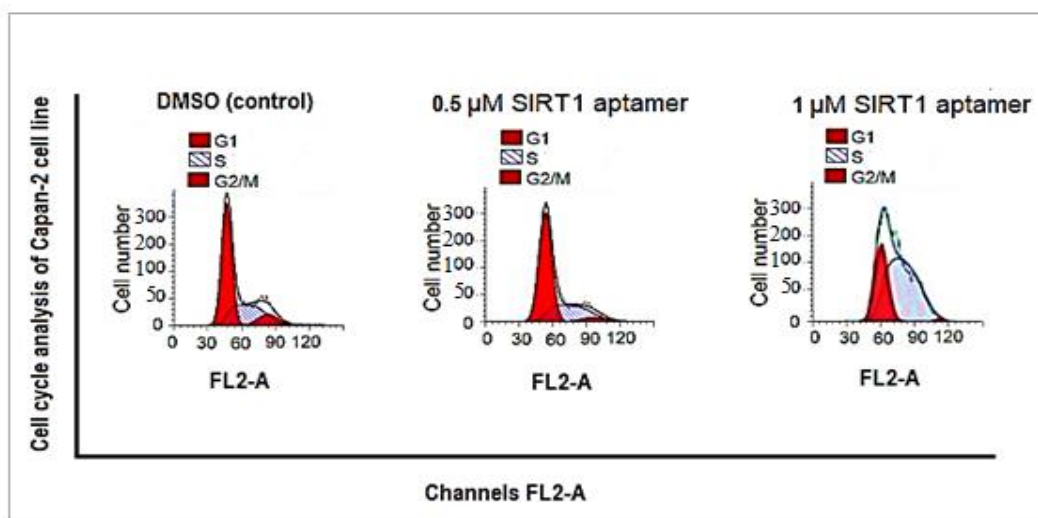
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1	3.00 ± 0.09	29.70 ± 2.13

Anticancer and apoptotic activities of SIRT1 activators through cell cycle arrest in Capan-2 human pancreatic carcinoma cell lines

1.5	3.00 ± 0.09	43.6 ± 3.16
2	3.00 ± 0.09	54.70 ± 2.37

To illustrate the effects of SIRT1 aptamer on pancreatic cancer cell growth, the cell cycle distribution was assessed by treating Capan-2 cell lines at low concentrations (0.5 and

significantly increased from 32.44% to 97.14% ($p < 0.0001$) as the Capan-2 cell lines were treated with 1 μM SIRT1 aptamer (Fig 3). These results suggested that 1 μM SIRT1 aptamer could arrest Capan-2 cell lines in the S phase in a dose-dependent manner. The S stage is the stage in which DNA replication occurs, therefore, we inferred that SIRT1



1 μM) of SIRT1 aptamer for 72 h as shown in Fig.2. Compared with the control group (DMSO), SIRT1 aptamer at the concentration of 0.5 μM ; the percentage of S phase cells was markedly increased from 32.44% to 48.13% ($p < 0.05$). Additionally, the G2/M phase cell contents were significantly decreased from 16.36% to 5.03%. Particularly, the percentage of S phase Capan-2 cells was considerably

Figure 2. Cell cycle analysis of Capan-2 cell line was performed by flow cytometric analysis after 0.5 and 1 μM SIRT1 aptamer and DMSO treatment. Capan-2 cell lines were fixed and stained with propidium iodide (PI). DNA content was detected by Flow cytometry.

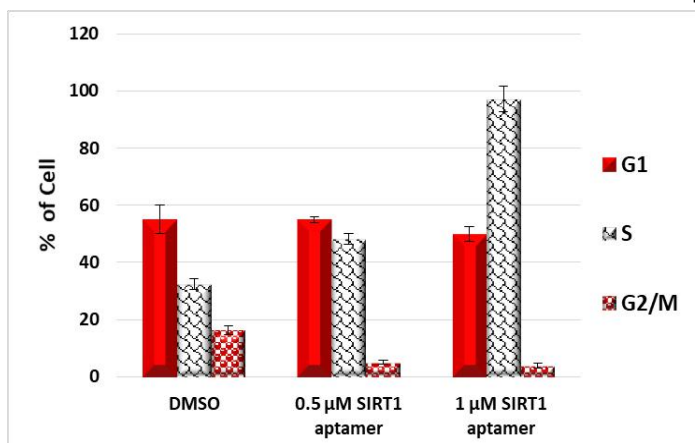
Figure 3. Distribution of cells at G1, S and G2/M phases of cell cycle after treatment with SIRT1 aptamer in Capan-2 cell line. Data are expressed as the mean \pm SD of three independent experiments: $p < 0.05$ and $p < 0.0001$ compared with the vehicle control (DMSO).

DISCUSSION

This study investigated the inhibitory mechanism of an anticancer drug for oligonucleotide aptamer. In doing so, the work in this study has provided information in greater detail regarding the use of apoptosis and cell cycle arrest assay to investigate the inhibitory effect of SIRT1 aptamers on pancreatic cancer cells (Capan-2). SIRT1 aptamer is currently being evaluated in preclinical studies as a potential cancer chemoprevention agent [13]. It has previously been shown to have anticancer activities in many types of pancreatic cancer cell lines aptamer [BxPc-3 (80%), Capan-2 (83%) and Aspc-1 (82.8%)] at 72h with IC_{50} 0.55, 0.5, 0.76 μM respectively. Importantly, SIRT1 aptamer showed no effect in cell viability on the non- pancreatic cancer cell line H6c7 [4]. Our study demonstrates the broad anticancer

aptamer could act on the course of DNA replication, which led to apoptosis. Of note, in 2 μM SIRT1 aptamer treated Capan-2 cell lines, 54% of the cells were undergoing apoptosis after 72h as detected in a prominent G1 apoptotic peak which confirmed using the Annexin V- FITC apoptosis assay.

properties of SIRT1 aptamer in Capan-2 cancer cell lines. SIRT1 aptamer caused a dose-dependent cancer cell growth inhibition, and this anticancer effect appears to be due to its ability to induce S-phase arrest and apoptotic cell death. The anticancer activities of SIRT1 aptamer probably reflect several mechanisms of action. In the present studies, the induction of apoptosis were observed within 72 h of treatment. A slight amount of apoptosis could be detected by low dose of SIRT1 aptamer treatment by flow cytometry using an annexin V- FITC/PI assay. After 72 h of treatment, SIRT1 aptamer prevented Capan-2 cell lines from entering the G1 phase of the cell cycle, resulting in the accumulation of cells in the S phase by 97.14%. These results had an agreement with other investigators which worked with other SIRT1 activators compounds called resveratrol in HL60



Anticancer and apoptotic activities of SIRT1 activators through cell cycle arrest in Capan-2 human pancreatic carcinoma cell lines

leukemia [14], U937 lymphoma [15], and CaCo-2 colon cancer cells [16]. Additionally, Researchers have suggested that SIRT1 activators induces S-phase arrest by decreasing the rate of DNA synthesis [17]. Subsequently, there are several ostensible mechanisms for the arrest in S phase induced by SIRT1 activators. In point of fact, SIRT1 activators has been demonstrates to suppress the activity of ribonucleotide reductase in L1210 cell lines [18], to block DNA synthesis as determined by [3H] thymidine incorporation in P-815 cell lines and K-562 cell lines [18], and may be prevent DNA polymerase in SV40 cell lines [19]. More studies are needed about the cellular mechanism of SIRT1 aptamer-mediated apoptosis in other pancreatic cancer cell lines. Furthermore, the SIRT1 aptamer-induced apoptosis of pancreatic cancer cells needs to be investigated in appropriate *in vivo* models.

CONCLUSION

The current study provides major information regarding SIRT1 aptamers effect on cell cycle of pancreatic cancer cells. The results presented here suggest that SIRT1 aptamer might be useful in the treatment of pancreatic cancer because its properties of a growth suppressor, in addition to its non-toxic property that discovered by scientists for specific killing of the tumor cells only avoiding unpleasant side effects from damage to the rest of the body.

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