Crosstalk Between Sirt1 Activators And Nf-kb Axis As A Therapeutic Target To Reduce Pancreatic Cancer

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ABSTRACT
The cellular availability of NAD+ is essential for Sir2uin 1 (SIRT1), a class III histone deacetylase, catalytic activity which make it as a reliable metabolic sensor. For evidence, SIRT1 had a high expression in tissue with metabolic activity including muscle, brain, liver, pancreas, and adipose tissue. Previous reports have documented the crosstalk between SIRT1 and nuclear factor kappa-B (NF-kB) signaling in conditions such as lymphoma, leukemia, and myeloma. The purpose of present study is to find the relationships between SIRT1 and NF-kB activation on pancreatic cancer cells (Capan-2, AsPC-1, and BxPC-3). We found the activation of NF-kB was inhibited by use 1 µM SIRT1 aptamer by increased the activity of SIRT1 protein. Overall, our findings suggested that SIRT1 activation resulting in inhibition of pancreatic cancer cells growth and significantly repression of NF-kB proteins activation, this activation of SIRT1 by aptamer may be considered as a potential future therapy for cancer especially pancreatic cancer.

Keywords: SIRT1, NF-kB p65 Transcription Factor, Pancreatic Cancer.

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INTRODUCTION
the SIRT1 ability to couple the chromatin structure of cell to its metabolic statues in a trusted cellular metabolic sensor [1]. This coupling lead to modulates gene transcription by histones and non-histone proteins modification [2]. Modification of non-histone protein by SIRT1 resulting in a different outcome including oxidative stress response, apoptosis, inflammatory and mitochondrial biogenesis response [3]. These diverse effects are yield a wide range non-histone protein SIRT1 targets including FOXO, p53, NF-kB, PGC1-α and PARP1 [3-7]. SIRT1 is mostly located inside the nucleus, however its location shift to cytosol through its two signals of nuclear exportation and its two signals of nuclear localization [9]. The subcellular localization of SIRT1 also affected by other factors such as cell and tissue type and as result to stimulus due to physiological and pathological conditions [9]. Numerous metabolic pathways are recruit SIRT1 such as glycolysis, gluconeogenesis, oxidative phosphorylation or urea cycle, fatty acid oxidation and synthesis [10,11]. Furthermore, SIRT1 also involved in many essential and homeostatic processes such as apoptosis, inflammation, mitochondrial biogenesis, [12] and tumorigenesis [9]. SIRT1 association with its non-histone substrate, p53, reveals its role in cancer development. This role is recently identified as a suppressor or promoter of tumor and determined by cell type and SIRT1 localization [9]. Therefore, SIRT1 regulation and expression was designated in many organs such as pancreas, muscle, brain, adipose tissue, or liver [12]. SIRT1 aptamer is a circular aptamer consist of 40 nucleotides (where the 5’ and 3’ ends are ligated) [13]. Aptamers are tiny and specific oligonucleotides [single-strand DNA (ssDNA) or RNA] and possess an intense binding affinity for the target protein [14]. Although SIRT1 role in cancers is still in debated, its specified to be an important target for aptamer in different human cancer cell lines models as anticancer effect including human pancreatic cancer (AsPC-1, Capan-2, BxPC-3), human adenocarcinoma of alveolar basal epithelial cells A549, estrogen-positive breast cancer MCF-7, and estrogen negative breast cancer MDA-MB- 468, children liver hepatocellular carcinoma HepG2, human bone osteosarcoma U2OS, human follicular thyroid cancer (FTC236, FTC238) and human colorectal adenocarcinoma Caco-2 [15-18]. The mechanism of how can SIRT1 aptamer inhibited the growth of cancer cell line is not studied until now. One of more mechanisms was suggested as shown in figure 1 that the increasing of SIRT1 enzyme activity leads to inhibiting NF-kB which can be blocked cytokine-induced NF-kB and led to downstream gene INOS [19].
**Figure 1:** The mechanism of SIRT1 aptamer to inhibition of NF-κB to lead to the reduced growth of cancer cell line. An ancient pathway was found for the SIRT1 and NF-κB where work in opposing control mechanisms in regulating of metabolic and inflammatory disorders in mammals [20]. It is approved that inhibition of NF-κB signaling by targeting SIRT1 to activate it was able to attenuate senescence-associated lung inflammation [21, 22]. NF-κB in mammals family consist of RelB, c-Rel, p65 (RelA), p105/p50 (NF-κB1), and p100/52 (NF-κB2) [23]. A study by Yeung et al. have illustrated the deacetylation activity SIRT1 on RelA/p65 subunit of NF-κB at lysine 310 and work as promoter-specific event to the sites of NF-κB on chromatin in lung cancer cells, non-small-cell [24]. The purpose of this study is investigated the mechanisms and relationships between SIRT1 and NF-κB activation on pancreatic cancer cells (Capan-2, AsPC-1, and BxPC-3). The results found that 1 μM SIRT1 aptamer was able to inhibit NF-κB activation of by increased the activity of SIRT1 protein. According to these results of SIRT1 aptamer mechanisms, it can be suggested that SIRT1 aptamer can be used in the future to the pancreatic cancer treatment.

**METHODS**

**Cell culture:** Cell lines of human pancreatic cancer: AsPC-1, Capan-2, and BxPC-3 were provided from American Type Culture Collection ATCC, H6c7 was provided from Kerafast, USA. McCoy’s 5A Medium was used to preserve Capan-2 cell line and Roswell Park Memorial Institute-1640 (RPMI-1640) medium was used to stabilize As PC-1 and BxPC-3 cell lines. 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. H6c7 cell lines were maintained in bovine pituitary extract + Keratinocyte SPM + EGF (Invitrogen). Then 1x antibiotic-antimycotic (Gibco) were added. 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, BxPC-3, and AsPC-1 were kept under sterile conditions. Five ml of phosphate buffered saline were added to wash the cell lines. Osmolarity of the cells were maintains by use a PBS solution. It act to balances the amount of salt ions inside the cell because it have a salt ions. 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. Then the supernatant was removed and the cell pellet underwent 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 [25].

**In vitro evaluation of the activity of SIRT1 enzyme in cells by Fluor de Lys Deacetylase assay:**

Through the use of Lys deacetylase kit with respect to manufacturer’s instructions, the measurement of deacetylase activity of SIRT1 was performed. Concisely, 4 x 10^8 cell lines was plated in 100 μl of appropriate media, after 24h the media was removed and 50 μl of medium containing 10 μl of (0.25, 0.5 and 1 μM from SIRT1 aptamer, 100 and 200 μM resveratrol, 100 μM suramin and 100 μM nicotinamide were incubated for 3h at 37°C before adding substrate. The reaction was set off via an addition of 25 μl of 2x Fluor de Lys substrate; Incubation of the plate was for 5hrs at 37°C, following by addition of 50μl of 1x Developer II/2 mM nicotinamide. After incubating for another 45mins at 37°C, the measurement of fluorescence was done by utilizing microplate-reading fluorimeter in the range 350-380 nm. Also emitted light was detected in the range 450-480 nm. Calculation of SIRT1 enzyme activity was done by deducting the background in the presence of suramin and resveratrol, an activator of SIRT1 activity [26].

**In vitro detection for the activity of NF-κB p65 Transcription Factor:**

Measurement of NF-κB (p65 subunit) capacity for its DNA-binding ability in the nuclear extracts of Capan-2, BxPC-3, and AsPC-1 cancer cell lines was done by using TransAMe NF-κB Kits and Nuclear Extract Active Motif, Carlsbad, CA, 92008, USA as shown by manufacturer’s instructions. In brief, in a 96-well plate the immunobilization of an oligonucleotide containing the NF-κB consensus binding site (5’-GGGACTTTCC-3’) has done. Specific bind was happen between this nucleotide and activated NF-κB in nuclear extracts. Accessible epitope on p65 due to bound NF-κB to its target DNA, was targeted by specific antibody then detect the NF-κB bound to the oligonucleotide. The qualification of reaction was done by densitometry where it visualized by adding a conjugated secondary antibody, horseradish peroxidase, which provide a sensitive colorimetric spectrum. The specificity of the assay where confirmed by adding a competitor, including non-immobilized consensus oligonucleotide or mutated consensus oligonucleotide. An arbitrary units were used to show the results (one unit is the DNA binding capacity and shown by 1 μg of whole cell extract from HeLa cells stimulated with TNF-a/μg proteins of BxPC-3, Capan-2, and AsPC-1 nuclear extract [27].

**RESULTS**

**In vitro evaluation of the activity of SIRT1 enzyme in pancreatic cell lines by Fluor de Lys Deacetylase assay:**

In this experiment, SIRT1 activity, was estimated on cell lines of Aspc-1, BxPc-3, and Capan-2 in parallel under the same conditions to investigate the mechanism of SIRT1 aptamer in cell lines. The measurement SIRT1 activity in BxPc-3 cell lines using SIRT1 aptamer at 0.25, 0.5 and 1μM. Then measure its activity when used 100μM resveratrol (SIRT1 activator control), 100 μM suramin and nicotinamide (SIRT1 inhibitor control). The result showed that SIRT1 activity increased with SIRT1 aptamer as compared with control resveratrol, suramin and nicotinamide as shown in figure (2).
Crosstalk Between Sirt1 Activators And Nf-κb Axis As A Therapeutic Target To Reduce Pancreatic Cancer

**Figure (2):** SIRT1 activity in BxPc-3 cell line. The results denote the mean ± SEM of 3 unique experiments carried out in triplicate, p< 0.005. (BAS aptamer = SIRT1 aptamer).

The results showed that the SIRT1 activity was increased in Capan-2 cell line when used SIRT1 aptamer at 1µM compared with high concentration 100µM resveratrol, and 100 µM Suramin and Nicotinamide as shown in figure (3).

**Figure (3):** SIRT1 activity in Capan-2 cell line. The results denote the mean ± SEM of 3 different testing done in triplicate. p< 0.0001. (BAS aptamer = SIRT1 aptamer).

Additionally, the measurement of SIRT1 activity in Aspc-1 cell line when treated with SIRT1 at 1µM caused activation of SIRT1 higher than 100µM resveratrol, Suramin and Nicotinamide as shown in figure (4).

**Figure (4):** SIRT1 activity in Aspc-1 cell line. The results represent the mean ± SEM of 3 different testing done in triplicate. p< 0.001. (BAS aptamer = SIRT1 aptamer).

**In vitro detection the activity of NF-κB p65 Transcription Factor**

Study the effect of SIRT1 aptamer on NF-kB activation were done by use ELISA-based assay to determine the capacity of NF-kB p65 subunit for DNA-binding in nuclear extracts of BxPC-3, Capan-2, and AsPC-1 cell lines (Figure 5). The cell lines presented a transcription factor constitutive activation. Adding a SIRT1 aptamer, at 1 µM induced biphasic changes in NF-kB. Binding activity of NF-kB was decrease to 150%, 130% and 130% compared to control 100%, P<0.001, at 8 h in cell lines of Bx-PC-3, Capan-2, and AsPC-1 respectively. The decrease was 180%, 145% and 140% of the control 100%, P<0.005 at 16 h in cell lines of Bx-PC-3, Capan-2, and AsPC-1 respectively. Arbitrary units/µg protein were used to express nuclear extracts BxPC-3, Capan-2, and AsPC-1 and are the mean ± SE of three independent observations. P<0.005 versus control.
Figure (5): show the capacity of NF-κB (p65 subunit) to binding DNA in nuclear extracts of cancer cell lines (BxPC-3, Capan-2, and AspC-1). P< 0.0005.

DISCUSSION
This study has investigated oligonucleotide SIRT1 aptamer as anticancer drug discovery. In doing so, the work in this study has provided information in greater detail regarding the evaluation of SIRT1 enzyme activity in pancreatic cell lines by Fluor de Lys Deacetylase assay and detection the activity of NF-κB p65 transcription factor to investigate the mechanism of relationship between SIRT1 and NF-κB on pancreatic cancer cells (BxPc-3, Capan-2, and AspC-1). Most studies have established that SIRT1 was involved in cancer cells such as Caco-2 [28], MCF-7, A549 [29], U20S [30], MDA-MB-468 [31] and HepG2 [32, 33]. In particular, many studies illustrated the role of SIRT1 in cancer cells, the effect of SIRT1 is varied from suppressor to an activator of cancer progression depending on the tissue [34]. Kabra, (2010) found that knockdown of SIRT1 raises the percentage of tumour advance by promoting cell spread, whereas over-expression of SIRT1 decreases tumour induction and expansion in nude mice [35]. This result is in agreement with other study that found the pharmacological activation of SIRT1 by SIRT1 aptamer caused reduction in cell viability of cell lines AspC-1, BxPc-3, and Capan-2 [15]. Figure 2-4 shows that treatment of pancreatic cancer cells with 1μM SIRT1 aptamer significantly increased the activity of SIRT1 in pancreatic cancer cells and these high level of SIRT1 activity caused to decreased NF-κB activation at 8h and 16h in the same pancreatic cancer cells. NF-κB has an ability to control the proliferation and the differentiation of cells, take part in inflammation processing and in immune response and in cancer pathway [36]. In about 95% of cancer types, there were activation of constitutive NF-κB [37-39]. In most pancreatic cancer, there was activation of NF-κB constitutively [40]. For instance, activation of constitutive NF-κB in pancreatic cancer of human cells play an important role in tumour cell lines of the pancreas [41]. The role of NF-κB in pancreatic cancer may explained in three lines. The first line suggests that the NF-κB activation constitutively is found in about 70% of human pancreatic cancer cell lines such as PANC-1 [42] BxPC-3 [43], and MIA PaCa-2 [44]. However, it showed to be not activated in non-tumorigenic epithelial cells and normal pancreatic cells or in immortalized. The second line of evidence for the NF-κB role in pancreatic carcinogenesis was illustrated by the study of many pancreatic cell lines and tumour models. NF-κB role of suppression of cancer elimination was dear by apoptosis restore in pancreatic cancer cells via DNA signalling activation [45]. For evidence, using inhibitors of NF-κB or lkbα super-repressor transfection enhances the doxorubicin or etoposide (VP16) apoptotic effects in pancreatic cancer cells resistant [46]. The third line of evidence suggests the NF-κB role in pancreatic carcinogenesis occurs at early steps. This evidence as shown by the ability of NF-κB to manipulate pathways contributing in initiating elements of cancer. For instance, the RAS oncogene [47] or resistance to apoptosis participates in invasive pancreatic cancer resistance [48]. In this research, we explore that activator of SIRT1 by SIRT1 aptamer as possible therapeutic treatment for BxPC-3, Capan-2, and AspC1 cancer cell lines pancreatic cancer diseases as 1μM SIRT1 aptamer can inhibited the NF-κB activation by increased the activity of SIRT1 protein.

CONCLUSION
According to the results of SIRT1 aptamer mechanisms, it can be suggested that SIRT1 aptamer can be used in the future to the pancreatic cancer treatment.

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REFERENCES
7. Rajamohan SB, Pillai VB, Gupta M, Sundaresan NR, Birukov KG, Samant S, Hottiger MO, Gupta MP. SIRT1
Crosstalk Between Sirt1 Activators And Nf-Kb Axis As A Therapeutic Target To Reduce Pancreatic Cancer


