Combination of Salermide and Cholera Toxin B Induce Apoptosis in MCF-7 but Not in MRC-5 Cell Lines

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Original Article

ABSTRACT

Background: *Sirtuin1* is an enzyme that deacetylates histones and several non-histone proteins including P53 during the stress. *P300* is a member of the histone acetyl transferase family and enzyme that acetylates histones. Hereby, this study describes the potency combination of Salermide as a *Sirtuin1* inhibitor and cholera toxin B (CTB) as a *P300* activator to induce apoptosis Michigan Cancer Foundation-7 (MCF-7) and MRC-5.

Methods: Cells were cultured and treated with a combination of Salermide and CTB respectively at concentrations of 80.56 and 85.43 μmol/L based on inhibitory concentration 50 indexes at different times. The percentage of apoptotic cells were measured by flow cytometry. Real-time polymerase chain reaction was performed to estimate the messenger ribonucleic acid expression of *Sirtuin1* and *P300* in cells. Enzyme linked immunosorbent assay and Bradford protein techniques were used to detect the endogenous levels of total and acetylated P53 protein generated in both cell lines.

Results: Our findings indicated that the combination of two drugs could effectively induced apoptosis in MCF-7 significantly higher than MRC-5. We showed that expression of *Sirtuin1* and *P300* was dramatically down-regulated with increasing time by the combination of Salermide and CTB treatment in MCF-7, but not MRC-5. The acetylated and total P53 protein levels were increased more in MCF-7 than MRC-5 with incubated combination of drugs at different times. Combination of CTB and Salermide in 72 h through decreasing expression of *Sirtuin1* and *P300* genes induced acetylation of P53 protein and consequently showed the most apoptosis in MCF-7 cells, but it could be well-tolerated in MRC-5.

Conclusion: Therefore, combination of drugs could be used as an anticancer agent.

Keywords: Apoptosis, cholera toxin B, Michigan Cancer Foundation-7, MRC-5, Salermide

INTRODUCTION

The histone acetyltransferases (*HATs*) induce transfer of acetyl group to lysine amino acid residues, which present

the histone protein tails from acetyl coenzyme A to form ε-N-acetyl lysine.[1] Therefore, they facilitate the accessibility of transcription factors to deoxyribonucleic acid (DNA).[2] Histone deacetylases (*HDACs*) comprise a super family of enzymes involved in regulating the lifespan, which include regulation of transcription.[3] On the other hand, the *HDACs*, despite *HATs*, cause increase in lifespan.[4] Class III *HDACs* were discovered more recently and this group of deacetylases was named "*Sirtuins*" (silent information regulators).[5] The *Sirtuins* have a nicotine adenine dinucleotide as a unique cofactor to this family that is necessary for the removal of the acetyl group from the lysine residues (deacetylases function).[6] *P300* is a member of the mammalian *HAT* protein family, which suggests that this molecule is competent of acetylating all core histone proteins and it is an important transcriptional co-activator, which may play a distinct role in regulation of a wide range of biological processes such as survival and apoptosis through histone acetylation.[7] Importantly, alteration of gene expression in cancer based on interaction of these epigenetic modifications (post-translation), play a significant role in tumorogenesis.[8] In diseases like cancer, often there occurs an imbalance between the expression of transcriptional co-activator proteins that contain *HATs* and *HDACs* families.[9] In human cancers, it has been shown that *HAT* activities were disrupted.[10] The *HATs* would often down-regulate and *Sirtuin1* often up-regulate in several types of tumors.[11,12] Histones are not the only proteins that can be alteration, *P300* and *Sirtuin1* presumably can also catalyze acetylation and deacetylation of several non-histone proteins such as P53 (the most important tumor suppressor gene. Activation of P53 can lead to cell cycle arrest, DNA repair and apoptosis.[13] Inactivation of *P300* mediates deacetylation of P53 and negatively regulates the activity of this protein.[14] *Sirtuin1* mediates deacetylation of P53 and negatively regulates the activity of this protein.[15] In normal cells, P53 is a short-lived protein due to activity of mouse double minute 2 homolog (Mdm2) as a ubiquitin ligase, to inhibit and destabilize P53, so P53 levels are undetectable and inactive to induce apoptosis.[16] In response to various types and stress levels, which cause DNA damage, *HATs* family mediate acetylation of P53 in C terminus and blocks some of the major P53 ubiquitination sites by Mdm2.[17] This function leads to P53 protein stabilization and activation of P53 protein in human cells.[18] Hyperacetylation of P53 can also cause the hyperactivity of this protein.[19] It seems that *P300* is able to acetylated and activate P53 and induce apoptosis in response to DNA damage in some cancer cells.[20] On the other hand, *Sirtuin1* is able to deacetylase and inhibit P53 activity and suppress the induction of apoptosis in a number of cancer cells.[21] The balance of P53 acetylation and deacetylation, respectively mediated by the *HATs* (particularly *P300*) and *HDACs* (particularly *Sirtuin1*), is usually well-regulated, but the balance often gets upset in diseases like cancer.[22] Studies suggest that pharmacologic inhibition of *Sirtuin1* may promote apoptosis by direct acetylation of P53 in some cells and can be used as an anticancer strategy.[23] Inactivation of *P300* and activation of *Sirtuin1* are encountered in several types of tumors such as in certain types of human cancers breast carcinomas.[24,25] The human breast carcinoma cell line Michigan Cancer Foundation-7 (MCF-7) has a wild-type P53, but this tumor suppressor gene is responsible for epigenetic event is not functional and cannot induce apoptosis.[26] These effects seem to be reversed in cancer cells by activation of *P300* and inactivation of *Sirtuin1*.[27,28] The studies suggest that pharmacologic activation of *P300* and inhibition of *Sirtuin1* may promote apoptosis by direct hyperacetylation of P53 in cancer cells and could be used as an anti-cancer strategy.[29,30] Salermide is a *Sirtuin1* inhibitor and cholera toxin B (CTB) is a small molecule activator of *P300*.[31,32] We know there are no such reports on effects combination of Salermide and CTB as an anti-tumor to induce P53 acetylation in cancer cell lines. We assumed that the apoptotic effect of these drugs is different in normal and cancer cells.[32,33] In this study, we investigated of apoptotic effects of combination Salermide as a *Sirtuin1* inhibitor and CTB as a *P300* activator to induce P53 protein acetylation and consequent apoptosis in MCF-7 and MRC-5 (lung fibroblasts as non-tumorigenic) cell lines.

METHODS

Cell lines, drug, treatment and culture condition

Human breast cancer MCF-7 and human lung fibroblasts MRC-5 were purchased from the

National Cell Bank of Iran-Pasteur Institute. CTB (N-[4-chloro-3-trifluoromethyl-phenyl]-2-ethoxy-benzamide), an activator of *P300* and Salermide (N-{3-[(2-hydroxynaphthalen-1-ylmethylene) -amino]-phenyl}-2-phenylpropionamidea) as the inhibitor of *Sirtuin1* were purchased from sigma (USA). All cell lines used in the present study were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (sigma) supplemented with 10% fetal bovine serum (sigma) and 1% penicillin-streptomycin (sigma) and incubated at 37°C and in a humidified atmosphere containing 5% CO_2. Drugs were dissolved in stock solutions and for treatments the compounds were diluted in dimethyl sulfoxide (DMSO) to the appropriate concentrations according to reported procedures.[16] After the cells were >80% confluent and growing exponentially in 10 cm diameter culture dishes, 10^5 cells (MCF-7 or MRC-5) were counted and plated in 3 cm diameter culture dishes and kept in RPMI-1640 culture medium for 24 h, which were then incubated with certain concentrations of CTB and Salermide combination, based on inhibitory concentration (IC50) index, at different times (24, 48 and 72 h).[35]

IC50 assay

The IC50 values for the CTB and Salermide in MCF-7 groups were acquired after 24 h of treatment. Briefly, 10^4 cells (MCF-7) were counted and placed into each well of a 24-well micro plate and were treated with various drugs concentrations (0, 6.25, 12.5, 25, 50, 100, 150, 200 μM doses) for 24 h and the Methyl Tiazolyl Tetrazolium (MTT) survival assay was then carried out for evaluating the cell viability with different drugs concentrations of MCF-7 groups. A graph of viability versus drugs concentration were used to calculate IC50 values for MCF-7 cell line.[2,27]

Flow-cytometric analysis

The percentage of apoptotic cells was measured by flow cytometry following AnnexinV flow cytometry1-histogram (FL1-H) and propidium iodide (PI) (FL2-H) labeling. A minimum of 5×10^5 cells/ml were analyzed for each sample. Cells were treated with a combination of CTB (85.43 μmol/L) and Salermide (80.56 μmol/L) for 24, 48 and 72 h and then washed in phosphate-buffered saline (PBS) and re-suspended in binding buffer (10×; 5 μl). AnnexinV-FITC was added to 195 μl cell suspensions and then analysis was carried out according to the manufacturer's protocol (BMS500F1/100CE AnnexinV-FITC, eBioscience, USA). Finally, the apoptotic cells were counted by FACScan flow cytometry (Becton Dickinson, Heidelberg, Germany). These experiments were carried out in triplicate and were, independently, repeated at least 3 times.[18,36]

Reverse transcription and real-time polymer chain reaction analysis

RT quantitative real-time polymerase chain reaction (Real time - PCR) was performed to quantitatively estimate the messenger ribonucleic acid (mRNA) expression of *P300* and *Sirtuin1* in MCF-7 and MRC-5 cells before and after treatment with a combination of CTB and Salermide at different times. Total RNA was isolated by RNeasy mini kit (Qiagen), treated by RNase-free DNase set (Qiagen) to eliminate the genomic DNA. The RNA concentration was determined using a biophotometer (Eppendorf). Total RNA (100 ng) was reverse-transcribed to complementary DNA (cDNA) by using the RevertAid™ first strand cDNA synthesis kit (Fermentas) according to the manufacturer's instructions. The maxima SYBR Green Rox qPCR master mix kit (Fermentas) was used for RT-PCR. Primer sequences are shown in Table 1. RT-PCR reactions were performed using step one plus (Applied Biosystem). The PCR amplification conditions consisted of 10 min at 95°C followed by 40 cycles of denaturation step at 95°C for 15 s and annealing and extension for 1 min at 60°C. Data were analyzed using the comparative Ct (ΔΔct) method. The relative expression level of *P300* and *Sirtuin1* were calculated by determining a ratio between the amount of *P300* and *Sirtuin1* and that of endogenous control. Melting curve analysis (60°C → 95°C increment of 0.3°C) was used to

Table 1: Primers used in real-time polymerase chain reaction

Primer ID	Primer sequences
P300-F	AGCAACCACAGCAGCAACTC
P300-R	GTCGTCTCAAGATGTCTCGGAAT
GAPDH-F	AAGCTCATTTCCTGGTATG
GAPDH-R	CTTCCTCTTGTGCTCTTG
$SIRT_1$-F	TGGCAAAGGAGCAGATTAGTAGG
$SIRT_1$-R	CTGCCACAAGAACTAGAGGATAAGA

GAPDH=Glyceraldehyde 3-phosphate dehydrogenase, as endogenous control, SIRT=Sirtuin

determine the melting temperature of specific amplification products and primer dimmers. These experiments were carried out in triplicate and were independently repeated at least 3 times.[37]

Bradford protein assay

Total (intracellular) protein concentration was determined by Bradford method. Bradford protein quantification assay is an accurate procedure for determining the concentration of protein in solution based on binding of Coomassie Blue dye to proteins. This method was carried out before enzyme linked immunosorbent assay (ELISA) assay. Total proteins extracted from MCF-7 and MRC-5 cells before and after combination of CTB and Salermide treatment will be described later in ELISA assay. Bovine serum albumin was used at 9 different concentrations (0.25, 0.5, 1, 1.5, 2, 3, 4, 5 and 6 mg/ml) to prepare a protein standard. After diluting the protein standards, the stock dye reagent was prepared (500 mg Coomassie Blue was dissolved in 500 ml methanol and was added to 100 ml phosphoric acid and 50 ml double-distilled water H_2O [ddH_2O]) that was diluted in 8 ml ddH_2O. A total of 2 ml of dye reagent was added to each tube of protein standard and was incubated at room temperature for at least 5 min. Absorbance of the protein standards and experimental samples were carried out by the spectrophotometry (Bausch and Lomb, Germany) at 595 nm and finally, a standard curve was plotted.[38]

Acetylated and total P53 sandwich ELISA assay

ELISA was used to specifically detect endogenous levels of total and acetylated P53 protein generation in MCF-7 and MRC-5 cells in the presence or absence combination of CTB and Salermide at different times (24, 48 and 72 h). Acetylated and total P53 ELISA Kit was prepared by cell signaling technology and cell lyses were prepared at the first step. Briefly, cells were harvested under treated conditions by the combination of CTB and Salermide at different times, media was removed and cells were washed with cold PBS. PBS was removed and 0.5 ml of ice-cold cell lyses buffer with 1 mM phenyl methyl sulfonyl fluoride was added to each plate and incubated on ice for 5 min. Cells were scraped off the plate and were transferred to an appropriate tube and a freeze-and-thaw test was performed 3 times. The tubes were micro-centrifuged at 4°C, for 10 min and the supernatant was transferred to a new tube. This supernatant was the cell lysates. For doing ELISA assay at first, the concentration of total protein extract in both cell lysates was determined using the Bradford assay. Sandwich ELISA was performed according to the manufacturer's protocol. Finally, the absorbance of samples were read in ELISA reader (Hyperion, Germany) at 450 nm wavelength and ELISA analysis was calculated based on control index. All experiments were carried out in triplicate.[39]

Statistical analysis

All the quantitative data were presented as the mean ± standard deviation. One-way analysis of variance (ANOVA) with *post-hoc* test was performed to determine the statistical significance among different groups by using the SPSS software package 16.0 IBM modeler (2009). Significance was accepted at a level of $P < 0.05$.

RESULTS

IC50

After the treatment of MCF-7 cells with MTT solution in this assay, the dark blue formazan crystals were seen in cells, which indicated their metabolic activity. The reduction in the number of cells was dependent on the cell type as shown by the half-maximal IC50 index. The IC50 values for the CTB and Salermide were established [Figure 1a and b]. The results showed that the essential CTB and Salermide concentration to achieve the IC50 in MCF-7 cells at 24 h respectively were 85.43 and 80.56 μmol/L [Figure 1a and b].

Flow-cytometry

To establish the anti-apoptosis potential of the combination CTB and Salermide, we first investigated the effects of this combination on the proliferation of the breast carcinoma cell line (MCF-7). The flow cytometry results showed that this combination drugs at different times (24, 48 and 72 h) could significantly induce apoptosis in MCF-7 cells and it was increased with ascending time ($P < 0.001$) [Figure 2a and b]. Combination drugs treatment arrested MCF-7 cell

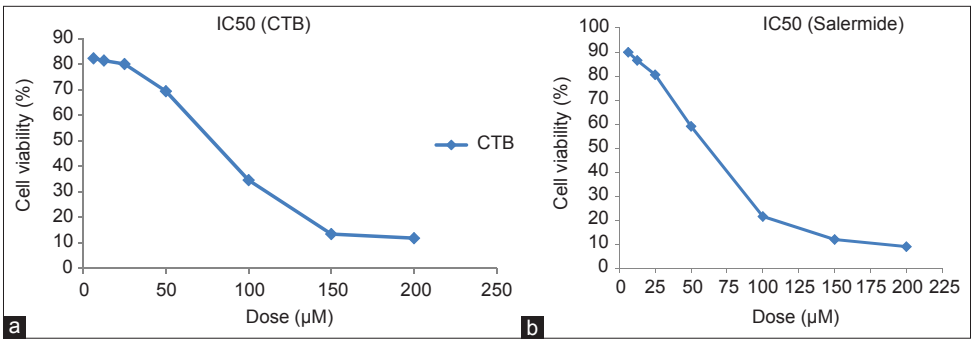

Figure 1: Inhibitory concentration (IC50) assay for half-maximal IC analysis of cholera toxin B (CTB). (a) And Salermide. (b) In MCF-7 cancer cell lines after 24 h of treatment. Cells were incubated with or without the CTB and Salermide using 0, 6.25, 12.5, 25, 50, 100, 150 and 200 μM doses and the relative amount of viable cells were estimated by measuring the absorbance of the cell suspension after incubation with MTT assay was carried out and a graph of viability versus drug concentration was used to calculate IC50 values for MCF-7cell line

proliferation (≥95% of inhibition) in 72 h, whereas its inhibition on MRC-5 cells proliferation in all different times were negligible; although, different times (24, 48 and 72 h) could significantly induce apoptosis in MRC-5 cells and it was increased with ascending time ($P > 0.05$) [Figure 2a and b]. MCF-7 apoptotic cells showed a sharp increase at all times in comparison with MRC-5 cells ($P < 0.001$). DMSO was used in the control sample (vehicle Drugs) a small amount of cell death in both cell lines at different times was observed ($P < 0.05$) [Figure 2a and b].

RT-PCR

We examined the inhibitory effects of Salermide and the activator effects of CTB in combination of drugs at different times on the mRNA expression of *Sirtuin1* and *P300* on MCF-7 and MRC-5 cells using RT-PCR. The *P300* gene expression was dramatically up-regulated by CTB treatment in combination of drugs with an ascending time in MCF-7 cells, in particular, at 72-h treatment its increased expression was significantly raised [Figure 3, $P < 0.001$]. The *Sirtuin1* gene expression was dramatically down-regulated by Salermide treatment in combination of drugs with time in MCF-7 cells [Figure 3, $P < 0.01$]. In MRC-5 cells, the expression of *P300* was increased 72 h after the CTB treatment ($P > 0.05$), but it was not statistically significant in different times ($P > 0.05$) [Figure 3] and the expression of *Sirtuin1* was also significantly reduced at all treatment times, but the difference in the expression among different times were not statistically significant ($P > 0.05$) [Figure 3]. However, in combination of drugs the effect of CTB treatment on up-regulation of *P300* and Salermide treatment on down-regulation of *Sirtuin1* expression were significantly higher in MCF-7 cells in comparison with MRC-5 cells [Figure 3, $P < 0.01$].

Acetylated and total P53 sandwich ELISA

To investigate further distinct effects of combination drugs on cell apoptosis, the ELISA analysis was conducted in MCF-7 (wild-type P53) and MRC-5 cells. The cells were treated at different times (0, 24, 48 and 72 h) with a combination of drugs, to study its effects on the acetylation status of the P53 as targets of *P300* and *Sirtuin1*. The results of ELISA analysis was calculated based on control index. The results showed that combination drugs could induce P53 acetylation in MCF-7 and MRC-5 cells and significantly increase in the total protein levels with ascending time until 48-h treatment in MCF-7 cells, but not in MRC-5 cells ($P < 0.05$). Interestingly, between 48 and 72 h, decrease of protein levels was observed in MCF-7 cells [Figure 4a]. Notably, after treatment by combination drugs at all different times, the acetylated P53 protein levels in MCF-7 cells was significantly higher than in MRC-5 cells ($P < 0.05$) [Figure 4a]. Consistently, we also performed the mentioned method to examine the total P53 protein levels in both cell lines. These results were similar to results of acetylated P53 except for an increase in the total P53 protein levels in MRC-5 cells until 48 h after treatment ($P < 0.05$) [Figure 4b]. In the control samples (using DMSO without combination

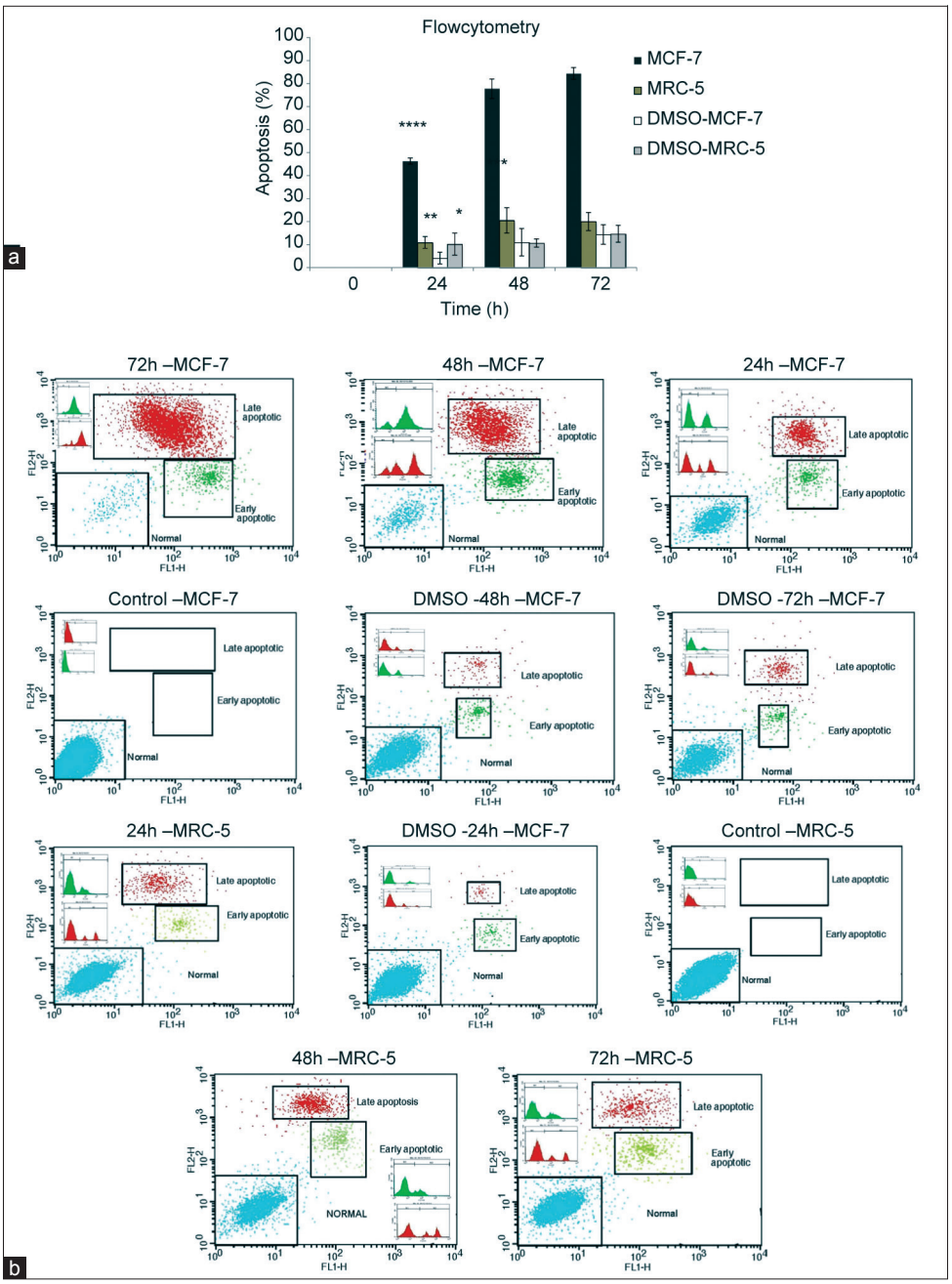

Figure 2: Relative levels of apoptotic cells in Michigan Cancer Foundation (MCF-7) and MRC-5 treated with combination of drugs for different times. Cells incubated with the vehicle dimethyl sulfoxide (DMSO) were used as a control. (a and b) The percentage of apoptotic cells was measured using the AnnexinV FITC (FL1-H) and propidium iodide (PI) (FL2-H) assay. ****$P < 0.001$ versus all other groups MCF-7 cells treated with combination of cholera toxin B and Salermide. ***$P < 0.05$ versus all other groups MRC-5 cells. **$P < 0.05$ versus all other groups MCF-7 cells incubated with the DMSO was used as a control. *$P < 0.05$ versus 48 and 72 h groups MRC-5 cells incubated with the DMSO were used as control. (b) Cells that are AnnexinV-positive and propidium iodide negative are in early apoptosis as phosphatidyl serine (PS) translocation has occurred; although, the plasma membrane remains intact. Cells that are positive for both AnnexinV and PI either are in the late stages of apoptosis or are already dead as PS translocation has occurred and the loss of plasma membrane integrity is visible

drugs), there was negligible effect on inducing total and acetylated P53 in both cell lines at the different times of study ($P > 0.05$) [Figure 4a and b].

DISCUSSION

The potencies and functional mechanisms of the combination drugs (CTB and Salermide) were

studied at the concentration that was confirmed by IC50 on the MCF-7 as breast cancer cell line and MRC-5 as non-tumorigenic control cells. In recent years, a few researchers have described the therapeutic effect of *P300* activation and inhibitor of *Sirtuin1* but there are no reports of their combination effects on the diverse types of cancerous and non-cancerous cells.[29,40] The role of *P300* and *Sirtuin1* during stress are complex and their activator and inhibitor effects are probably cell context-specific.[41,42] Until date, no clear explanation exists about molecular mechanisms of the combination of *P300* activators (particularly CTB) and *Sirtuin1* inhibitors (particularly Salermide) in different cells or about comparing their effects on cancerous and normal cells at different times. It is notable that in this study, the apoptotic potency of combination drugs was examined on the MCF-7 because expression level of *P300* and *Sirtuin1* are respectively down-regulated and up-regulated, P53 is wild-type and this kind of cancer is the most prevalent malignancy in woman.[43] In this study, treatment by the combination of CTB and Salermide at various time showed a time-dependent increase in apoptotic cell count of the cancerous cells, but not in non-tumorigenic MRC-5 cells as measured by flow-cytometric assay. On the other hand, although CTB and Salermide could effectively induce activation of *P300* and inhibition of *Sirtuin1* and subsequent cancer cell death it did not have such an effect on fibroblastic cells (poor cell apoptosis). These results revealed that probable apoptotic sensitivity of MRC-5 cells to CTB and Salermide stimulation was negligible, which agreed with the previous findings of Lara *et al.*[4] showed that Sirtuin inhibitors can induce massive apoptosis in cancerous, but not in non-malignant cells. The severe apoptotic effect in the MCF-7 was observed after 72 h of incubation with CTB and Salermide, so as approximately 4% of MCF-7 cells, were viable while at this time MRC-5 cells showed only a slight increase in level of apoptosis (only about 25%). It seems that this result is because of the presence of the

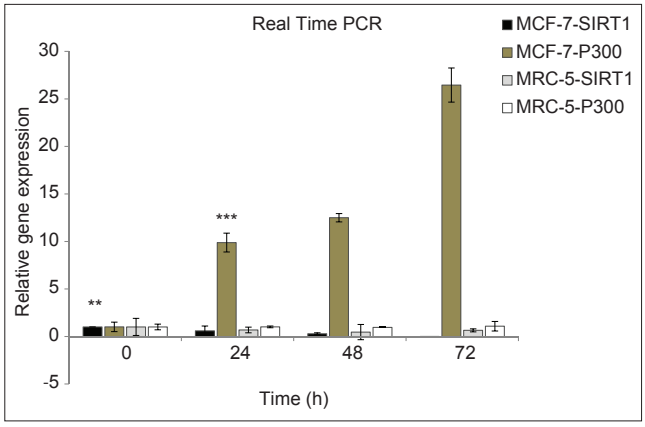

Figure 3: Results of real-time quantitative polymerase chain reaction before and after combination of *cholera toxin* B and Salermide at different times on the *P300* and *Sirtuin1* messenger ribonucleic acid expression in Michigan Cancer Foundation (MCF-7) and MRC-5 cells. Relative expression levels of each gene were obtained by using the comparative Ct (ΔΔct) method. Histone acetyltransferases activator and *Sirtuin1* inhibitor-caused epigenetically activated *P300* and inhibited *Sirtuin1*. Values were the means of triplicate experiments ***$P < 0.001$ versus and **$P < 0.05$ versus control (non-treated drugs) and other MCF-7 groups. No significant difference was seen in other groups

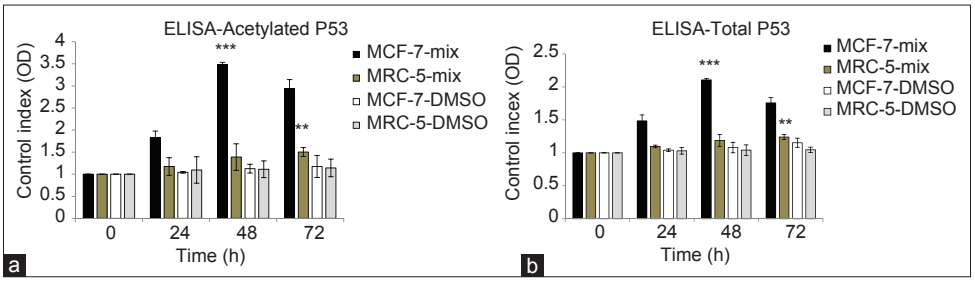

Figure 4: The results of enzyme linked immunosorbent assay analysis based on the control index for acetylated and total P53 protein generated in Michigan Cancer Foundation (MCF-7) and MRC-5 cells were treated with and without drugs at different times. Cells were treated with combination of drugs for 0, 24, 48 and 72 h. Values are mean + standard error of triplicate experiments. (a) ***$P < 0.001$ versus all other groups in different time. **$P < 0.05$ versus acetylated P53 in MCF-7 with combination of drugs treatment at 72 h. (b) *$P < 0.001$ versus all other groups in different time. **$P < 0.05$ versus total P53 in MRC-5 without drugs treatment and with drugs treatment at time 48 and 72 h. No significant difference was shown in total and acetylated P53 content of other groups

wild-type P53. Our results were supported by the findings of Janknecht[44] which shows modulation of *P300* function may consider novel therapies directed against tumors with wild-type P53. Therefore, it was assumed that in MCF-7 cells, up-regulation of *Sirtuin1* and degradation of *P300* expression promoted cell survival and combination of CTB and Salermide could induce apoptosis in these cancer cells with wild-type P53 in a time-dependent manner. Our results were matched with the findings of Zou *et al*.[1] They state that trichostatin A (HDAC inhibitor) can induce apoptosis in BGC-823 gastric cell line in a time-dependent manner. In addition, our results were consistent with the findings of Vempati *et al.*[7] and Molvaersmyr *et al.*[41] who demonstrated that *P300* may be an important regulator of wild-type P53 function and *P300* fails to acetylated mutant P53. It was observed that combination of CTB and Salermide was ineffective to promote acetylation of P53 and it seemed that it cannot activate P53 to induce apoptosis in fibroblastic cells. Once it was determined that combination of CTB and Salermide anti-tumor activity was primarily because of the promotion of apoptosis, we decided to study the molecular mechanisms involved in this process. We first studied the expression of *P300* and *Sirtuin1* in combination of CTB and Salermide-mediated apoptosis by using RT-PCR. The results of RT-PCR assay indicated that Salermide mediated inhibition of *Sirtuin1* expression in a time-dependent manner and CTB was responsible for the over-expression of *P300* in a time-dependent manner in MCF-7 cells (progressive increase in *P300* mRNA levels after 24, 48 and 72 h) by exposure to CTB. This was in accordance with the findings of Mantelingu *et al.*[27] which showed that incubation of *P300* with increasing concentration of CTBP results in a dose-dependent enhancement of *P300* HAT activity by *HAT* assay. It was found that *P300* expression level in MRC-5 cells by the treatment of CTB slightly increase in a time-dependent manner only in 72 h. No alteration of *P300* expression levels between the other groups of (non-treatment, 24 and 48 h) MRC-5 samples (normal *P300* expression) was found. We also found that *Sirtuin1* expression levels in MRC-5 cells was low and after treatment with Salermide in combination of drugs slightly decreased in a time dependent manner. Importantly, we found that in the non-cancerous cells, *Sirtuin1* silencing due to Salermide was at least equivalent to MCF-7 breast cancer cells. Subsequently, we observed that *Sirtuin1* silencing occurred particularly after 48 h of Salermide treatment in MCF-7 cells. More importantly, it was discovered that in the non-cancerous cells, *P300* expression may increase because CTB was at least equivalent to MCF-7 breast cancer cells. Subsequently, we observed that strong *P300* transcription occurred particularly after 48 h of CTB treatment in MCF-7 cells. However, *Sirtuin1* silencing and relative stability of *P300* expression until 48 h of treatment and only little enhancement after 72-h incubation was not effective on the cell apoptosis and viability of the non-cancerous cells. Importantly, it seemed that low levels of apoptosis, which was observed in all MRC-5 samples after treatment with a combination of CTB and Salermide, was more significantly relevant to apoptotic effects of DMSO as carrier and solvent of drugs on fibroblastic cells. This was in agreement with the findings of Ikushima and Miyazono[45] that Smad complex and transforming growth factor beta (TGF-β) recruit co-activators such as *P300* to induce growth arrest and/or apoptosis through P53 protein interaction in cancerous cells and loss of *P300* genes could lead to tumor progression, which in contrast with normal human epithelial cells seemed to be refractory to *P300* activation and *Sirtuin1* silencing (in the absence of applied stress, *Sirtuin1* silencing induces growth arrest and/or apoptosis in human epithelial cancerous. Inversely, these observations disagree with the findings of Bedford *et al.*[46] that knockdown and lack of *P300* gene suppress cell growth and increase apoptosis effect in the cancerous cells. The results indicated that function of *P300* and *Sirtuin1* were different in MCF-7 and MRC-5 cells and that *Sirtuin1* inhibition and *P300* activation might enable MCF-7 cancer cell apoptosis, but it seems non-essential for the apoptosis of lung fibroblast cells. These results were also similar to the study of Karamouzis *et al.*[47] that the down-regulation of *P300* expression was observed in breast cancer cells and *P300* may provide a tumor suppressor-like function although the tumor-suppressor function of *P300* is still unclear. Also, similar to the study of Kojima *et al*.[48] who noted that the up-regulation of *Sirtuin1* expression was observed in breast cancer

MCF-7 cells and that treatment with a *Sirtuin1* inhibitor, Sirtinol, induced inhibition of *Sirtuin1* expression and subsequently inhibited cell growth in human prostate cancer cell lines. These results were dissimilar with the findings of Fermento *et al.*[24] which showed up-regulation of *P300* expression in murine mammary adenocarcinoma LM3 cells. Our results showed that the effect of 24 and 48 h of treatment of MRC-5 cells by CTB in combination of drugs on the expression of *P300* mRNA were similar to the non-treatment condition. Subsequently, we used RT-PCR to evaluate *P300* and *Sirtuin1* expression in both cell lines before treatment. We observed lower expression of *P300* and over-expression of *Sirtuin1* in MCF-7 cells in comparison to MRC-5 cells, which might be explained by the fact that decrease of *P300* and increased level of *Sirtuin1* expression in MCF-7 cells leads to inhibited apoptosis and mediated survival in response to stress. Therefore, these results suggest that *P300* and *Sirtuin1* keep a balance of specific acetylation levels for proper cellular function and mediate survival in normal cells. This finding was in accordance with the previous findings of Peck *et al.*[25] that indicate that the degree of acetylation is largely mediated by a balance between *HATs* and HDAC in normal cells. Our findings indicate that stimulation of cell death by CTB and Salermide requires respectively the activation of *P300* and *Sirtuin1* genes, showing the combination of drugs potential anti-tumor effect. This observation, similar to a recent study by Chen *et al.*[49] has showed that small molecule activators of *P300* may act as anti-cancer agents. To investigate further and determine the total and acetylated status of P53 in response to the combination of CTB and Salermide in the cells, ELISA analysis was performed after ensuring the level of total protein concentration using the Bradford method. We found a remarkable increase in P53 acetylation level in a time-dependent manner until 48 h in MCF-7 but not in MRC-5 cells. Consistently, combination of drugs induced a similar increase of total P53 in both cell lines as proposed earlier. In MRC-5 cells, we saw a little increase of total and acetylated P53 protein levels in a time-dependent manner of combination CTB and Salermide treatment compared with MCF-7 cells, which showed CTB over-expression of *P300* and Salermide lower-expression of *Sirtuin1* in combination of drugs resulted in up-regulation of acetylated P53 and subsequently P53 activation in MCF-7 cells (based on previous results by flow-cytometry and RT-PCR assay). Different researchers reported various data about how *P300* activation and inhibition of *Sirtuin1* could induce (Dornan *et al.*[10] and Lain *et al.*[50]) or not induce (Vempati *et al.*[7] and Ota *et al.*[30]) P53 acetylation in cancer cell lines. A direct correlation between total and acetylated P53 protein levels and combination of CTB and Salermide toxicity in the MCF-7 cell line was discovered. These results suggested that incubation of MCF-7 with a combination of CTB and Salermide might induce hyperacetylation of P53 protein and apoptosis in MCF-7 cells. Our results indicated that slight decrease of total and acetylated P53 was evident at 72-h incubation in MCF-7 cells. We suggested that although an increase in total and acetylated P53 levels in response to *P300* activation and *Sirtuin1* inhibition at this time was accorded, P53 protein was undetectable by ELISA assay due to the release of proteases and degradation process inside the cancer cells after 48 h of cell death. A decrease in total P53 level in response to drugs incubation after 48 h in the control MRC-5 cells was not observed, which indicated that combination of drugs could not induce significant apoptosis even after 48 h in MRC-5 cells. These observations showed that although in MCF-7 cells, P53 was wild-type it was a target for deacetylation of *P300* and acetylation of *Sirtuin1* and so could not induce apoptosis due to this aberrant epigenetic event. These data were in accordance with the findings of Iyer *et al.*[51] that *P300* contributes to maintain P53 stability by regulating its ubiquitination and P53 acetylation (activate P53 function) may promote the P53-*P300* complex (epigenetic changes). Furthermore in accordance with the findings of Pruitt *et al*.[52] who reported that *Sirtuin1* is a cancer-related gene and inhibits P53 function through epigenetic changes. Our findings suggested that acetylation of wild-type P53 as a tumor suppressor might lead to activation of apoptotic program and was integral to cytotoxic activity of the combination drugs to induce massive apoptosis in less than 24 h of treatment in MCF-7 cells. This finding further highlights our theoretical assumptions, indicating that activation of *P300* and inhibition of *Sirtuin1* is required for the induction of cell death and P53

acetylation in cancer cells only for wild-type P53 and combination of CTB and Salermide triggers neither cell death nor P53 acetylation in normal cells. Our results are similar to the previous study of Gu and Roeder[53] that indicates a novel pathway for wild-type P53 acetylated by co-activator, *P300* to induce apoptosis in some cell types. Kamel *et al*.[54] suggested that Sirtuin inhibitors are targets for *Sirtuin1* and Sirtuin$_2$ and P53 acetylation is important for the cell death induction. We suggested that combination of *P300* activators, such as CTB and inhibitors of *Sirtuin1* like Salermide, may function through common pathways and mediate their cytotoxic effects through targeting P53 and its acetylation.

CONCLUSIONS

We formulated the hypothesis that dysfunction of *P300* and up-regulation of *Sirtuin1* could plays crucial roles in inhibiting pro-apoptotic protein expression in cancer cells, so it seems that P53 aberrantly repressed in MCF-7 cells. Thus, the treatment with a combination of CTB and Salermide could predominantly induce apoptosis through enhancing activity of *P300* and inhibition of *Sirtuin1* and consequent hyperacetylation and reactivation of tumor suppressor P53 to induce cell death in MCF-7 cancer cells. We described that combination of CTB and Salermide as an activator of *P300* and inhibitor of *Sirtuin1* could make a promising novel class of agents to the future anti-tumorigenic drugs that target acetylation of proteins; thus might be a target for cancer therapy.

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