Original Article
Middle East Journal of Cancer 2013; 4(4): 163-173

The Apoptotic Effects of the P300 Activator on Breast Cancer and Lung Fibroblast Cell Lines

Mohammad Reza Salahshoor*, Cyrus Jalili*♦, Ahmad Shebanizadeh Darehdori, Mozafar Khazaei*, Shiva Roshankhah*, Rostam Ghorbani***

**Fertility and Infertility Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran*
***Department of Anatomical Sciences and Molecular Biology,Rafsanjan University of Medical Sciences, Rafsanjan, Iran*

Abstract

Background: P300 is an enzyme that acetylates histones during stress. It also acetylates several non-histone proteins, including P53 which is the most important tumor suppressor gene. P53 plays an important role in the apoptosis of tumor cells. Hereby, this study describes the potency of cholera toxin B subunit as a P300 activator to induce apoptosis in a breast cancer cell line (MCF-7) and a lung fibroblast cell line (MRC-5) as a non-tumorigenic control sample.

Methods: MCF-7 and MRC-5 were cultured in RPMI-1640 and treated with or without cholera toxin B subunit at the concentration of 85.43 µmol/L, based on the half-maximal inhibitory concentration index at different times (24, 48 and 72 h). The percentage of apoptotic cells was measured by flow cytometry. Real-time quantitative RT-PCR was performed to estimate the mRNA expression of P300 in MCF-7 and MRC-5 with cholera toxin B subunit at different times. We used the ELISA and Bradford protein techniques to detect levels of total and acetylated P53 protein generated in MCF-7 and MRC-5.

Results: Our findings indicated that the cholera toxin B subunit effectively and significantly induced more apoptosis in MCF-7 compared to MRC-5. We showed that expression of P300 up-regulated by increasing the time of the cholera toxin B subunit treatment in MCF-7 but not in MRC-5. In addition, the acetylated and total P53 protein levels increased more in MCF-7 cells than in MRC-5 cells.

Conclusion: Cholera toxin B subunit induced significant cell death in MCF-7, but it could be well tolerated in MRC-5. Therefore, cholera toxin B subunit can be suggested as an anti-cancer agent.

Keywords: Apoptosis, P300 activator, Breast cancer, Lung fibroblast

♦Corresponding Author:
Cyrus Jalili, PhD
Fertility and Infertility Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran
Tel:+98-9188317220
Email:cjalili@yahoo.com

Introduction

Histone acetyl transferases (HATs) are enzymes that play pivotal roles in the regulation of gene expression

Received: July 7, 2013; Accepted: August 2, 2013

involved in acetylation. They act as transcriptional co-activators.[1] The HATs induce acetyl group transfer into lysine amino acid residues, which present the histone protein tails from acetyl CoA to ε-N-acetyl lysine. Therefore, they associate with open chromatin sequences that facilitate the accessibility of transcription factors to DNA (transcriptional activation).[2] Histone deacetylases (HDACs) in addition to HATs cause an increase in lifespan. However, they cooperate with acetylation and deacetylation in order to regulate transcription. They remove acetyl groups from histone tails thus preventing the attachment of transcription factors.[3] Importantly, alteration of gene expression in cancer based on interaction of these epigenetic modifications (post-translation) with each other plays a significant role in tumorigenesis.[4] In diseases such as cancer, often an imbalance occurs between expressions of transcriptional co-activator proteins. These proteins contain HAT and HDAC families.[5] P300 is a member of the mammalian HAT protein family with HAT activity which suggests that this molecule is competent for acetylating all core histone proteins and is an important transcriptional co-activator. P300 may play a distinct role in the regulation of a wide range of biological processes such as cell growth, proliferation, survival and apoptosis through histone acetylation.[6] In several diseases such as some human cancers, it has been shown that HAT activities are disrupted, which is often associated with malignant alterations.[7] The HATs often down-regulate and become inactivated in several types of tumors.[8] However, histones are not the only proteins that can be acetylated; P300 presumably can also catalyze acetylation of several non-histone proteins such as P53 (the most important tumor suppressor gene).[9] P53 has been shown to be functionally regulated by acetylation and its stability changes.[10] More than half of human tumors often have mutations in P53.[11] Alterations in tumor suppressor genes are not always due to mutations; they may also be due to this sort of epigenetic alteration.[12] The P53 tumor suppressor protein plays a major role in cellular response to DNA damage and other genomic aberrations.[13] Activation of P53 can lead to cell cycle arrest, DNA repair, and apoptosis.[14] Following DNA damage, human P53 acetylates at Lys382.[15] Inactivation of P300 mediates deacetylation of P53 and negatively regulates the protein's activity.[16] In normal cells, P53 is a short-lived protein as a result of the activity of Mdm2 (a negative regulator), as a ubiquitin ligase[17] to inhibit and destabilize P53. Therefore P53 levels are undetectable and inactive to induce apoptosis. In response to various types and levels of stress that causes DNA damage, the HAT family mediates acetylation of P53 at the C terminus and some of the major P53 ubiquitination sites are blocked by Mdm2.[18] This function leads to P53 protein stabilization and activation in human cells.[19] Hyperacetylation of P53 can also cause the hyperactivity of this protein.[20] Possibly P300 is able to acetylate, activate P53, and induce apoptosis in response to DNA damage in some cancer cells.[21] The balance of P53 acetylation (positively regulates P53 activity) and deacetylation (negatively regulates P53 activity) mediated by the HATs (particularly P300) and HDACs, respectively is usually well-regulated. However, this balance often fails in diseases such as cancer.[22] Inactivation of P300 is observed in several tumor types in certain types of human cancers, including breast carcinomas.[23] Breast cancer is the most common cancer among females and is the second leading cause of death among women worldwide.[24] Each year about 1.15 million cases of female breast cancer are diagnosed worldwide, while about 502,000 die from the disease.[25] Some reports suggest that inactivation of P300 probably mediates P53 deacetylation, inhibits P53, and possibly mediates apoptosis in response to various types of stress in some of the malignancies.[26] The human breast carcinoma cell line MCF-7 has a wild-type P53 but this tumor suppressor gene is responsible for the epigenetic event (hypoacetylation P53), is not functional and cannot induce apoptosis.[27] These effects seem to be reversed in cancer cells when P300 is

activated.[28] The studies suggest that pharmacologic activation of P300 may promote apoptosis by direct hyperacetylation of P53 in cancer cells, which can be used as an anti-cancer strategy. CTB is the only known small molecule activator of HATs that is specific to P300 by alteration of the P300 structure[29], and is synthesized from salicylic and anacardic acids. CTB goes into the hydrophobic compartments of the P300 enzyme and leads to modification of the α-helix. It also results in the modification of ring structured amino acids within the histone tail of the HAT domain, which is presumably responsible for the activation of P300 activity.[30] However, only a small number of small molecules have been recognized to affect the P300 gene and the CTB drug, thus far. As far as we know, there are no such reports on the effects of CTB as an anti-tumor agent or about its effects on HAT P300 (activity of P300) to induce P53 acetylation in cancer cell lines. In contrast, inhibitors of HDACs have been extensively studied as therapeutic targets.[31] We have assumed that, based on HDAC inhibitors, the pharmacologic activation of P300 may promote apoptosis by inducing direct acetylation of P53 in some cancer cells. Also, we have presumed that it has different effects on inducing apoptosis in normal and cancer cells. Thus, it could be used as an anti-cancer strategy.[32] In this study, we investigated the apoptotic effects of CTB as the activator of P300 in P53 protein acetylation. We also studied the consequent apoptosis in MCF-7 (breast adenocarcinoma) and MRC-5 (lung fibroblasts as non-tumorigenic control sample) cell lines.

Materials and Methods

Cell lines, drug, treatment and culture conditions

Human breast cancer MCF-7 and human lung fibroblasts MRC-5 were purchased from the National Cell Bank of Iran at Pasteur Institute. N-(4-chloro-3-trifluoromethyl-phenyl)-2-ethoxy-benzamide (CTB), an activator of P300, was purchased from Sigma (C6499, USA). All cell lines used in the present study were cultured in RPMI-1640 medium (Sigma) supplemented with 10% fetal bovine serum (FBS, Sigma) and 1% penicillin-streptomycin (Sigma), and incubated at 37°C in a humidified atmosphere that contained 5% CO_2. CTB was dissolved in stock solutions and the compounds were diluted in DMSO to the appropriate concentrations for treatments according to previously reported procedures.[17] After the cells reached >80% confluency and grew exponentially in 10 cm diameter culture dishes, 105 cells (MCF-7 or MRC-5) were counted and plated in 3 cm diameter culture dishes and maintained in RPMI-1640 culture medium for 24 h. Cells were then incubated with determined concentrations of CTB according to the half-maximal inhibitory concentration (IC_{50}) index at different times (24, 48 and 72 h). Photographs of cultures were taken before and after treatment with CTB at different times under an inverted

Table 1. Primers used in real-time PCR.

Primer ID	Primer sequences
P300-F	AGCAACCACAGCAGCAACTC
P300-R	GTCGTCTCAAGATGTCTCGGAAT
GAPDH-F	AAGCTCATTTCCTGGTATG
GAPDH-R	CTTCCTCTTGTGCTCTTG

GAPDH = Glyceraldehyde 3-phosphate dehydrogenase, as housekeeping gene

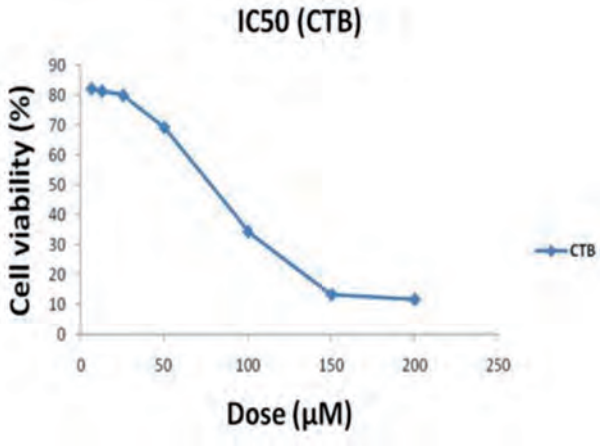


Figure 1. IC_{50} assay for half-maximal inhibitory concentration analysis of the cholera toxin B subunit (CTB) in MCF-7 cancer cell lines after 24 h of treatment. Cells were incubated with or without the CTB using 0, 6.25, 12.5, 25, 50, 100, 150 and 200 µM doses. The relative amounts of viable cells were estimated by measuring the absorbance of the cell suspension after incubation with MTT assay. A graph of viability versus drug concentration was used to calculate IC_{50} values based on the trend line equation (Y=-0.421X+85.97) for the MCF-7 cell line (85.43µM).

microscope (Nikon, TE 2000-U, Japan).[33]

Half-maximal inhibitory concentration (IC_{50}) assay

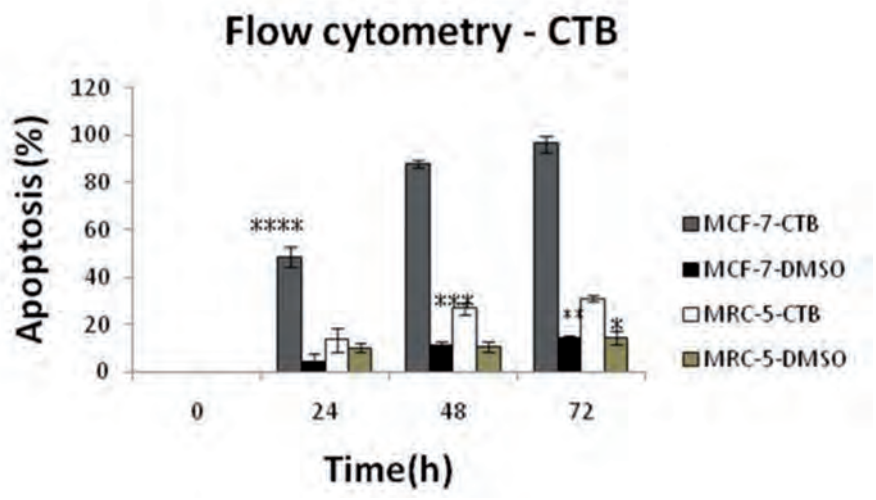
The IC_{50} values for the CTB in MCF-7 groups were acquired after 24 h of treatment. Briefly, 104 cells (MCF-7) were counted and placed into each well of a 24-well micro plate, then treated with various drug concentrations (0, 6.25, 12.5, 25, 50, 100, 150, and 200 µM) for 24 h. We performed the MTT survival assay to evaluate cell viability with different drug concentrations of the MCF-7 groups. A graph of viability versus drug concentration was used to calculate IC50 values for the MCF-7 cell line.[2,28]

Flow cytometric analysis

The percentage of apoptotic cells was measured by flow cytometry following Annexin V (FL1-H) and PI (FL2-H) labeling. A minimum of 5×10^5 cells/ml were analyzed for each sample. Cells were treated with CTB (85.43µmol/L) for 24, 48 and 72h, washed in PBS and re-suspended in binding buffer (1×; 5µl). Annexin V-FITC was added to 195µl cell suspension after which analysis was performed according to the manufacturer's protocol (BMS500F1/100CE Annexin V-FITC, eBioscience, USA). Finally, the apoptotic cells were counted by FACS can flow cytometry (Becton Dickinson, Heidelberg, Germany). These experiments were carried out in triplicate and independently repeated at least three times.[12,19]

Reverse transcription and real-time PCR analysis

We used real-time quantitative RT-PCR to quantitatively estimate mRNA expression of P300 in MCF-7 and MRC-5 cells before and after treatment with CTB at different times. Total RNA was isolated by the RNeasy Mini Kit (Qiagen) and treated by the RNase-free DNase set (Qiagen) to eliminate any genomic DNA. The RNA concentration was determined using a biophotometer (Eppendorf). Total RNA (100 ng) was reverse-transcribed to cDNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's instructions. The Maxima SYBR Green Roxq PCR Master Mix Kit (Fermentas) was used for real-time RT-PCR. Primer sequences are shown in Table 1. Real-time PCR reactions were performed using Step One Plus (Applied Biosystems). The PCR amplification conditions consisted of 10 min at 95°C followed by 40 cycles of a denaturation step at 95°C for 15 sec and annealing and extension for 1 min at 60°C. Data were analyzed by the Comparative Ct (ΔΔct) method. The relative expression level of P300 was calculated by determining the ratio between the amount of P300 and that of endogenous control. The melting curve analysis (60°C→95°C increment of 0.3°C) was used to determine the melting temperature of specific amplification products and primer dimers. These experiments were carried out in triplicate and independently repeated at least three times.[34]



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 of CTB at different times (24, 48 and 72h). The Acetylated and Total P53 ELISA Kits were prepared by Cell Signaling Technology and cell lyses were preparedat the first step. Briefly, cells were harvested under treated conditions by CTB at different times, media was removed, and cells washed with cold PBS. PBS was removed and 0.5ml of ice-cold cell lyses buffer plus 1mM phenyl methyl sulfonyl fluoride (PMSF) were added to each plate, followed by incubation on ice for 5 min. Cells were scraped off the plate and transferred to an appropriate tube. A freeze-and-thaw test was performed three times. The tubes were micro-

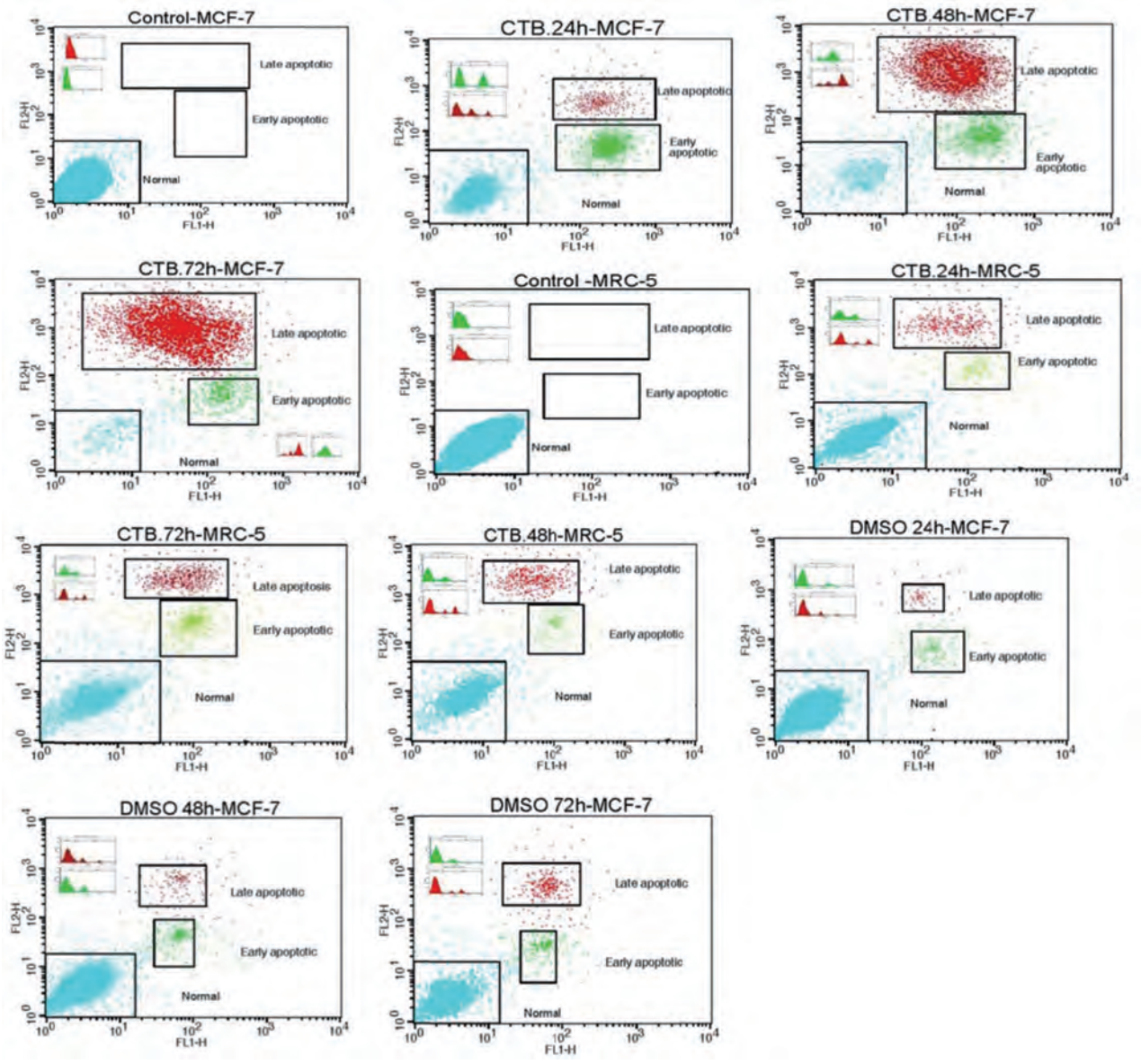


Figure 2. Relative levels of apoptotic cells in MCF-7 and MRC-5cells treated with 85.43µM/L of CTB for different times. Cells incubated with the vehicle (DMSO) were used as a control. A. The percentage of apoptotic cells was measured using AnnexinV FITC and PI assay. ***P<0.001 vs. all other groups of MCF-7 cells treated with cholera toxin B subunit (CTB).**P<0.05 vs. 0, 24 and 48 h groups of MRC-5 Cells. *P<0.05 vs. all other groups of MCF-7 cellsincubated with the vehicle (DMSO). B. MCF-7and MRC-5 cells were treated with 85.43 µM/L of CTB; Apoptosis was measured by flow cytometry following AnnexinV (FL1-H) and propidium iodide (PI;FL2-H) staining. Cells that areannexinV-positive and PI negative are in early apoptosis, as phosphatidylserine (PS) translocation hasoccurred, although the plasma membrane remains intact. Cells that are positive for both AnnexinV and PI are either in the late stages of apoptosis, as PS translocation has occurred and the loss of plasma membrane integrity is visible.

centrifuged at 4°C for 10 min and the supernatant was transferred to a new tube. This supernatant contained the cell lysates. To perform the ELISA assay, at first the concentration of total protein extract in both cell lysates was determined by the Bradford assay. Sandwich ELISA was performed according to the manufacturer's protocol. Finally, samples' absorbances were read in an ELISA reader (Hyperion, Germany) at 450 nm wavelength and ELISA analysis was calculated based on the control index. All experiments were carried out in triplicate.[36]

Statistical analysis

All quantitative data were presented as mean±standard deviation. One-way analysis of variance (ANOVA) with LSD post-hoc test was performed to determine the statistical significance among different groups using SPSS software package 16. $P<0.05$ was considered significant.

Results

Half-maximal inhibitory concentration (IC_{50})

After treatment of MCF-7 cells with MTT solution in this assay, dark blue form azan crystals were seen in the cells, which was indicative of their metabolic activity. The reduction in the number of cells was dependent on the cell type as shown by the IC_{50} index. The IC_{50} values for CTB were established (Figure 1). The results showed that the essential CTB concentration to achieve the IC_{50} in MCF-7 cells at 24h was 85.43 µmol/L (Figure 1).

Flow cytometry

To establish the anti-apoptosis potential of CTB, we first investigated the effects of this P300 activator on proliferation of the breast carcinoma cell line (MCF-7). The flow cytometry results showed that the 85.43 µmol/L concentration of CTB significantly induced apoptosis in MCF-7 cells and increased with ascending time based on the IC50 index at different times (24, 48 and 72h; $P<0.001$; Figures 2A and 2B). CTB treatment arrestedMCF-7 cell proliferation (≥95% inhibition) in 72 h, whereas its inhibition on MRC-5 cell proliferation at all time points was negligible. P300 increased apoptosis in MRC-5 with ascending time ($P>0.05$; Figures 2A and 2B). MCF-7 apoptotic cells showed a sharp increase at all time points compared with MRC-5 cells ($P<0.001$). DMSO was used in the control sample (vehicle CTB) and a small amount of cell death was observed in both cell lines at different times ($P<0.05$; Figures 2A and 2B).

Real-time PCR

It was suggested earlier that apoptotic induction in cancer cell lines by CTB requires the activation of P300 gene expression. To examine this hypothesis, we used two cell lines, MCF-7 as the cancerous cell line and MRC-5as the non-cancerous cell line. We examined the inhibitory effects of 85.43 µmol/L CTB (based on the IC_{50} index) on mRNA expression of P300 in MCF-7 and MRC-5 cells at different times using RT-PCR. P300 gene expression was dramatically up-regulated by CTB treatment with ascending time in MCF-7 cells; in particular, its increased expression was significantly raised at 72-h treatment ($P<0.01$; Figure 3). In MRC-5 cells, the expression of P300 also increased 72 h after CTB treatment ($P>0.05$), but it was not statistically significant at different times ($P>0.05$; Figure 4). However, the effect of CTB treatment on the up-regulation of P300 expression was significantly higher in MCF-7cells compared with MRC-5 cells ($P<0.01$, Figure 3).

Acetylated and total P53 sandwich ELISA

To investigate additional distinct effects of CTB (P300 activator) on cell apoptosis, we conducted an ELISA analysis in MCF-7 (wild-type p53) and MRC-5 cells. The cells were treated at different times (0, 24, 48 and 72h) with 85.43 µmol/L CTB to study its effects on the acetylation status of P53 as a target of P300. The results were calculated based on the control index. The results showed that CTB could induce P53 acetylation in MCF-7 and MRC-5 cells and significantly increase 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 72h, decreased protein level was observed in MCF-7 cells (Figure 4A). Notably, after treatment by CTB at all time points, there were significantly higher acetylated P53 protein levels in MCF-7 compared to MRC-5 cells ($P<0.05$; Figure 4A). Consistently, we performed the mentioned method to examine the total P53 protein levels in both cell lines. These results were similar to the results of the acetylated P53 except for an increase in total P53 protein levels in MRC-5 cells until 48h after treatment($P<0.05$; Figure 4B). In the control samples (using DMSO without CTB), there was a negligible effect on inducing total and acetylated P53 in both cell lines at different study time points ($P>0.05$; Figures 4A and 4B).

Discussion

The potencies and functional mechanisms of the recently developed P300 activator (CTB) were studied in MCF-7 as a breast cancer cell line and MRC-5 as non-tumorigenic control cells at the confirmed IC50 concentration. The result of CTB IC_{50} generally matched with the previously published data of Mantelingu et al.[28] about the effect of CTBP on P300 activation. Recent reports have presented that this compound can specifically enhance P300 activity in mammalian cells.[29] In recent years, a few researchers have described the therapeutic effect of P300 activation, but there are no reports about its effects on diverse types of cancerous and non-cancerous cells.[28,29] The role of P300 during stress is complex and its activator effect is probably cell context-specific.[37,38] It should be noted that in this study, the apoptotic potency of CTB was examined on MCF-7 because the expression level of P300 is down-regulated, P53 is the wild-type, and this type of cancer is the most prevalent malignancy in women. In this study, treatment by 85.43µmol/L of CTB at various times 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 could effectively induce the activation of P300 and subsequent cancer cell death, it did not have such an effect on fibroblastic cells (poor cell apoptosis). These results revealed that the probable apoptotic sensitivity of MRC-5 cells to CTB stimulation was negligible, which confirmed the previous findings by Fermento et al.[23] The increasing activity of P300 by TGF-β in normal skin and lung fibroblasts cells induces stimulation of collagen synthesis in fibrotic lesions. Iyeret al.[39] have shown that activities of P300 among mammary tumors and normal mammary glands differ. Thus, inactivation of P300 is confirmed in breast carcinoma and other solid tumors. This result appears to be due to the presence of wild-type P53. Our results have been supported by the findings of Janknechtet al.,[38] which showed increasing of P300 function may consider novel therapies directed against tumors with wild-type P53. Therefore, it was assumed that in MCF-7 cells, degradation of P300 expression promoted cell survival and CTB alone could induce apoptosis in these cancer cells with wild-type P53 in a time-dependent manner. Our results were in line with the findings of Isharwal et al.[5] who showed that increased P300 expression correlated with the fate of cancerous cells and has a prognostic value in predicting biochemical recurrence-free survival or apoptosis in tumor cells. The results of the current study were

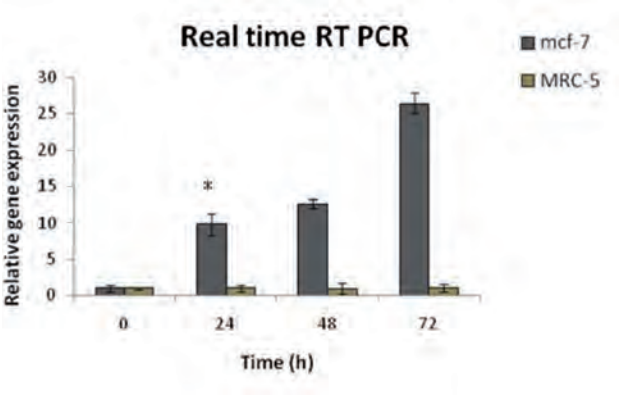


Figure 3. Results of real-time quantitative PCR before and after cholera toxin B subunit (CTB) at different times on the P300 mRNA expression in MCF-7 and MRC-5 cells. Relative expression levels of each gene were obtained using the comparative Ct (ΔΔct) method. HAT activator-caused epigenetically activated P300 values were the means of triplicate experiments.**$P<0.001$ vs. control (non-treated CTB) and other MCF-7 groups. No significant difference was seen in the other groups.

consistent with the findings of Vempati et al.[6] and Kristin et al.[37] who demonstrated that P300 might be an important regulator of wild-type P53 function and P300 failed to acetylate mutant P53. CTB anti-tumor activity has been shown to primarily be the result of apoptosis promotion. Real-time PCR assay results indicated that CTB was responsible for 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) via exposure to CTB. This was in accordance with the findings of Mantelingu et al.[28] Incubation of P300 with increasing concentrations of CTBP resulted in dose-dependent enhancement of P300 HAT activity according to the HAT assay. P300 expression level in MRC-5 cells by the treatment of CTB slightly increased in a time-dependent manner in only 72 h. No alteration of P300 expression levels was found between the other groups (non-treatment, 24 and 48h) of MRC-5 samples (normal P300 expression). More importantly, P300 expression might increase in non-cancerous cells because CTB was, at least, equivalent to MCF-7 breast cancer cells. Subsequently, we observed that strong P300 transcription occurred particularly after 48h of CTB treatment in MCF-7 cells. However, the relative stability of P300 expression until 48h of treatment and only slight enhancement after 72-h incubation did not affect cell apoptosis and viability of non-cancerous cells. It seemed that low levels of apoptosis, which were observed in all MRC-5 samples after treatment with CTB, showed a more significant association with the apoptotic effects of DMSO as a carrier and solvent of CTB in fibroblastic cells. This result agreed with the findings of Ikushima et al.[40] and Polley et al.[11] who have shown that Smad complex and TGF-β recruit co-activators such as P300 to induce growth arrest and/or apoptosis through P53 protein interaction in cancerous cells. Also, loss of P300 genes could lead to tumor progression, which seems to be refractory to P300 activation in contrast with normal human epithelial cells. Inversely, these observations disagreed with the findings of Chen et al.,[41] Bedfordet al.[42] and Goodman[43] who reported that knockdown and lack of the P300 gene suppressed cell growth and increased the apoptotic effect in cancerous cells. The results indicated that the function of P300 was different in MCF-7 and MRC-5 cells and P300 activation might enable MCF-7 cancer cell apoptosis, but it seemed non-essential for the apoptosis of lung fibroblast cells. These results were also similar to those of studies conducted by Karamouzis et al.[44] and Chan et al.[26] who observed down-regulation of P300 expression in breast cancer cells. However, these results contrasted the findings of Fermento et al.,[23] who showed up-regulation of P300 expression in murine mammary adenocarcinoma LM3 cells. Our results revealed that the effect of 24 and 48-h treatments of MRC-5 cells by CTB on the expression of P300 mRNA was similar to the non-treatment

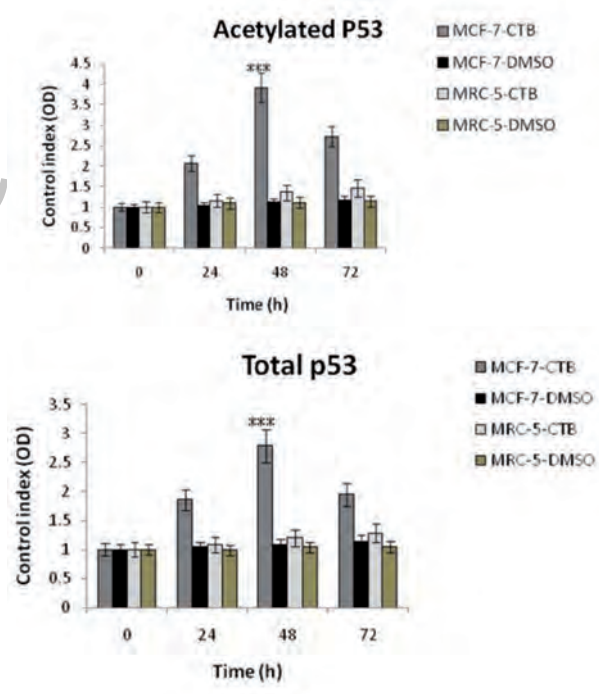


Figure 4. Results of ELISA analysis based on the control index for total and acetylated P53 proteins generated in MCF-7 and MRC-5 cells that were treated with and without cholera toxin B subunit (CTB) at different times. Cells were treated with 85.43 µmol/L of CTB for 0, 24, 48 and 72 h. Values are mean+SE of triplicate experiments. (A) **P*<0.001 vs. all other groups at different times. ** *P*<0.05 vs. total P53 in MRC-5 with salermide treatment at 72 hours. (B) **P*<0.001 vs. all other groups in different times. ***P*<0.05 vs. total P53 in MRC-5 without CTB treatment and with CTB treatment at times 48 and 72 h. No significant difference was shown in total and acetylated P53 content of other groups.

condition. We observed elevated P300 mRNA levels after 48 h of treatment. Subsequently, we used real-time PCR to evaluate P300 expression in both cell lines before treatment. There was lower expression of P300 in MCF-7 cells compared to MRC-5 cells, which might be explained by the fact that decreased P300 expression in MCF-7 cells led to inhibited apoptosis and mediated survival in response to stress. Therefore, these results suggested that P300 and HDACs maintained a balance in specific acetylation levels for proper cellular function and mediated survival in normal cells. This finding was in line with the previous findings of Peck et al.[36] that indicated the degree of acetylation was largely mediated by a balance between HAT and HDAC in normal cells. Our findings indicated that stimulation of cell death by CTB required activation of the P300 gene. This observation, similar to a recent study by Chen et al.[45] has shown that small molecule activators of P300might act as anti-cancer agents. We found a remarkable increase in P53 acetylation levels in a time-dependent manner until 48h in MCF-7 but not in MRC-5 cells. Consistently, CTB induced a similar increase of total P53 in both cell lines, as proposed earlier. In MRC-5 cells there was a slight increase in total and acetylated P53 protein levels in a time-dependent manner with CTB treatment compared to MCF-7 cells. CTB over-expression of P300 resulted in up-regulation of acetylated P53 and subsequently P53 activation in MCF-7 cells according to previous results by flow-cytometry and real-time PCR assay. Different researchers have reported various data about how P300 activation can induce or not induce P53 acetylation in cancer cell lines.[6,7] There was a direct correlation observed between total and acetylated P53 protein levels and CTB toxicity in the MCF-7 cell line. These results suggested that incubation of MCF-7 with CTB might induce hyperacetylation of the P53 protein and apoptosis in MCF-7 cells. Our results showed a slight decrease of total and acetylated P53 at 72-h incubation in MCF-7 cells. We suggested that, although increase in total and acetylated P53 levels in response to P300 activation 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 48h of cell death. There was no decrease observed in total P53 level in control MRC-5 cells in response to CTB incubation after 48h, which indicated that CTB could not induce significant apoptosis in MRC-5 cells after 48h. These observations showed that although the wild-type P53 was present in MCF-7 cells, it was a target for deacetylation and dysfunction of P300, thus P53 could not induce apoptosis due to this aberrant epigenetic event. This data was in line with the findings of Iyer et al.[39] that showed P300 contribution to the maintenance of P53 stability by regulation of its ubiquitination and P53 acetylation. Our findings suggested that acetylation of wild-type P53 as a tumor suppressor might lead to the activation of apoptotic program and was integral to cytotoxic activity of CTB to induce massive apoptosis in MCF-7 cells at less than 24h of treatment. Our results were similar to those of the previous study carried out by Gu et al.[46] that indicated a novel pathway for wild-type P53 acetylate by co-activator P300 to induce apoptosis in some cell types. Cui et al.[47] showed that Lys-CoA as an inhibitor of P300 has anti-proliferative activity against tumor cell lines, which contrasted our findings. We suggested that P300 activators such as CTB might function through common pathways and mediate their cytotoxic effects via targeting P53 and its acetylation. This was similar to the results of the study by Gauthier et al.[49] in which they reported that treatment of HT-29 cells (HIV-1) with forskolin and adenylatecyclase activated CBP (homologous gene of P300) which lead to new strategies for reducing virus production in HT-29 cells through P300 (CBP) activation.

Conclusion

We have described that CTB, as an activator of P300, can make a promising novel class of agents for future anti-tumorigenic drugs that target acetylation of proteins, which are epigenetically

activated by P300 and thus maybe a target for cancer therapy.

Acknowledgements

We would like to express our appreciation to Dr. Mohammad Kazemi, Dr. Nafiseh Esmaeili, and Dr. Marjan Gharagozloo for their sincere help without which this study could not be performed.

References

1. Zou XM, Li YL, Wang H, Cui W, Li XL, Fu SB, et al. Gastric cancer cell lines induced by Trichostatin A. *World J Gastroenterol* 2008;14:4810-5.
2. Giahuai T, Shundong C, Yuehua M, Richard LP, Delong L. Novel histone deacetylase inhibitors in clinical trials as anti-cancer agents. *Hematol Oncol* 2010;3:15-28.
3. Lara E, Mai A, Calvanese V, Altucci L, Lopez P, Chantar ML, et al. Salermide, a sirtuin inhibitor with a strong cancer-specific proapoptic effect. *Oncogene* 2009;28:781-91.
4. Dworkin AM, Hung TH, Toland AE. Epigenetic alteration in breast: Implication for breast cancer detection, prognosis and treatment. *Semin Cancer Biol* 2009;19:165-71.
5. Isharwal S, Miller MC, Marlow C, Makarov M, Partin AW, Veltri RW. P300 (histone acetyl transferase) biomarker predicts prostate cancer biochemical recurrence and correlates with changes in epithelia nuclear size and shape. *Prostate* 2008;68:1097-104.
6. Vempati RK, Jayani RS, Notani D, Sengupta A, Galande S, Haldar D. P300-mediated acetylation of histone H3 lysine 56 functions in DNA damage response in mammals. *J Biol Chem* 2010;285:28553-64.
7. Doran D, Shimuizo H, Perkins N, Hupp T. DNA-dependent acetylation of P53 by the transcription coactivators P300. *J BiolChem* 2003;278:13431-41.
8. Emanuele S, Lauricella M, Tesoriere GN. Histone deacetylase inhibitors: Apoptotic effects and clinical implications. *Int J Oncol* 2008;33:637-64.
9. Cazzalini O, Perucca P, Savio M, Necchi D, Bianchi L, Stivala LA, et al. Interaction of P21 with PCANA regulates the histone acetyltransferase activity of p300 in nucleotide excision repair. *Nucleic Acids Res* 2008;36:1713-22.
10. Huanh W, Chen C. Akt phosphorylation of P300 at Ser-1834 is essential for its histone acetyltransferases and transcriptional activity. *Mol Cell Biol* 2005;25:6592-602.
11. Polley S, Guha S, Roy NS, Kar S, Sakaguchi K, Chuman Y, et al. Differential recognition of phosphorylated transactivation domains of P53 by different p300 domains. *J Mol Biol* 2008;376:8-12.
12. Kim HJ, Bae SC. Histone deacetylase inhibitors: Molecular mechanisms of action and clinical trials as anti-cancer drugs. *Am J Transl Res* 2011;3:166-79.
13. Cazzalini O, Perucca P, Savio M, Necchi D, Stivala LA, Ducommun B, et al. Interaction of P21CDKN1A with PCNA regulates the histone acetyltransferases activity of P300 in nucleotide excision repair. *Nucleic Acids Res* 2008;36:1713-22.
14. Ozaki T, Nakagawara A. P53: The attractive tumor suppressor in the cancer research field. *J Biomed Biotechnol* 2011;10:11-23.
15. Leeuwen I, Lain S. Sirtuins and P53. *Cancer Res* 2009;9:230-55.
16. Cang S, Ma Y, Liu D. New clinical developments in histone deacetylase inhibitors for epigenetic therapy of cancer. *J Hematol Oncol* 2009;2:22.
17. Liu G, Chen X. Regulation of the P53 transcriptional activity. *J Cell Biochem* 2006;97:448-58.
18. Robbins SL, Kumar V. Robbins and cotran pathologic basis of disease. 7th ed. Vol. 11. Elsevier: Saunders; 2010:19-54.
19. Gregoretti IV, Lee YM, Goodson HV. Molecular evolution of the histone deacteylase family: Functional implications of phylogenetic analysis. *J Mol Biol* 2004;338:17-31.
20. Olmosa Y, Brosensb JJ, Lama EW. Interplay between SIRT proteins and tumor suppressor transcription factors in chemotherapeutic resistance of cancer. *Drug Resist Update* 2011;14:35-44.
21. Chim CS, Wong AS, Kwong YL. Absence of P300 gene promoter methylation in acute leukemia. *Cancer Genet Cytogenet* 2004;150:164-7.
22. Escande C, Chini CS, Nin V, Dykhouse KM. Deleted in breast cancer-1 regulates SIRT1 activity and contributes to high-fat diet-induced liver steatosis in mice. *J Clin Invest* 2010;120:545-58.
23. Fermento ME, Gandini NA, Lang CA, Perez JE. Intracellular distribution of P300 and its differential recruitment to aggresomes in breast cancer. *Exp Mol Pathol* 2010;88:256-64.
24. BoniolM, Gavin A, Vatten LJ. Breast cancer mortality in neighboring European countries with different levels of screening but similar access to treatment: Trend analysis of who mortality database. *BMJ* 2011;343:d441.
25. Montazeri A, Vahdaninia M, Harirchi I, Harirchi AM, Sajadian A, Khaleghi F, et al. Breast cancer in Iran: Need for greater women awareness of warning signs and effective screening methods. *Asia Pac Fam Med* 2008;7:6.
26. Chan HM, Thangue NB. p300/CBP proteins: HATs for transcriptional bridges and scaffolds. *J Cell Sci* 2001;114:2363-73.
27. Dworkin AM, Huang TH, Toland AE. Epigenetic alterations in the breast: Implications for breast cancer detection, prognosis, and treatment. *Semin Cancer*

Biol 2009;19:165-71.
28. Mantelingu K, Kishore AH, Balasubramanyam K, Pavan Kumar GV, Altaf M, Swamy SN,et al. Activation of P300 histone acetyltransferases by small molecules altering enzyme structure: Probed by surface-enhanced raman spectroscopy. *J Phys Chem B* 2007;111:4527-34.
29. Devipryia B, Paraneswari AR, Rajalakshmi G, Palvannan T, Kumaradhas P, et al. Exploring the binding affinities of P300 enzyme activators CTBP and CTB using docking method. *Indian J Biochem Biophys* 2010;47:364-9.
30. Wong P, Pickard A, Mccance DJ. P300 alters keratinocyte cell growth and differentiation through regulation of p21(Waf1/CIP1). *PLoS One* 2010;5:e8369.
31. Cen Y. Sirtuins inhibitors: The approach to affinity and selectivity. *Biochem Biophys Acta* 2010;1804:1635-44.
32. Calvanese V, Fraga MF. Sirt1 brings sternness closer to cancer and aging. *Aging (Albany NY)* 2011;3;162-7.
33. Gallinari P, Di MS, Jones P, Pallaoro M, Steinkuhler C. HDACs, histone deacetylation and gene transcription: From molecular biology to cancer therapeutics. *Cell Res* 2007;17:195-211.
34. Yang T, Fu M, Pestell R, Sauve AA. SIRT1 and endocrine signaling. *Trends Endocrinol Metab* 2006;17:186-91.
35. Jones AP, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 2002;3:415-28.
36. Helweg B, Gatbonton T, Schuler AD. Antitumor activity of a small molecule inhibitor of human silent information regulator 2 enzymes. *Cancer Res* 2006;66:4368-77.
37. Molversmyr AK, Sxther T, Gilfillan S, Lorenzo PI. A SUMO-regulated activation function controls synergy of c-Myb through a repressor–activator switch leading to differential P300 recruitment. *Nucleic Acids Res* 2010;38:4970-84.
38. Janknecht R. The versatile functions of the transcriptional coactivators p300 and CBP and their roles in disease. *Histol Histopathol* 2002;17:657-68.
39. Iyer NG, Ozdag H,Caldas C. p300/CBP and cancer. *Oncogene* 2004;23:4225-31.
40. Ikushima H, Miyazono K. TGFβ signalling: A complex web in cancer progression. *Nat Rev Cancer* 2010;10:415-24.
41. Chen LF, Greene WC. Regulation of distinct biological activities of the NF-kappaB transcription factor complex by acetylation. *J Mol Med (Berl)* 2003;81:549-57.
42. Bedford DC, Kasper LH, Fukuyama T, Brindle PK. Target gene context influences the transcriptional requirement for the KAT3 family of CBP and P300 histone acetyltransferases. *Epigenetics* 2010;5:9-15.
43. Goodman RH, Smolik S. CBP/P300 in cell growth, transformation and development. *Genes Dev* 2000;14:1553-77.
44. Karamouzis MV, Konstantinopoulos PA, Papavassiliou AG. Roles of CREB-binding protein (CBP)/P300 in respiratory epithelium tumorigenesis.*Cell Res* 2007;17:324-32.
45. Chen S, Feng B, George B, Megan CR. Transcriptional co-activator P300 regulates glucose-induced gene expression in endothelial cells. *Am J Physiol Endocrinol Metab* 2010;298:E127-37.
46. Gu W, Roeder RG. Activation of P53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. *Cell* 1997;90:595-606.
47. Cui L, Miao J, Furuya T, Fan QI, Li X, Rathod PK, et al. Histone acetyltransferases inhibitor Anacardic acid causes changes in global gene expression during in vitro plasmodium falciparum development. *Eukaryot Cell* 2008;7:1200-10.
48. Gauthier S, Tremblay MJ. Cholera toxin inhibits HIV-1 replication in human colorectal epithelial HT-29 cells through adenylatecyclase activation. *Antiviral Res* 2010;88:207-16.