

RESEARCH ARTICLE

Trichostatin A-induced Apoptosis is Mediated by Krüppel-like Factor 4 in Ovarian and Lung Cancer

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Abstract

Background: The histone deacetylase (HDAC) inhibitor trichostatin A (TSA) is known to mediate the regulation of gene expression and antiproliferation activity in cancer cells. Krüppel-like factor 4 (klf4) is a zinc finger-containing transcription factor of the SP/KLF family, that is expressed in a variety of tissues and regulates cell proliferation, differentiation, tumorigenesis, and apoptosis. It may either function as a tumor suppressor or an oncogene depending on genetic context of tumors. **Aims:** In this study, we tested the possibility that TSA may increase klf4 expression and cancer cell growth inhibition and apoptosis in SKOV-3 and A549 cells. **Materials and Methods:** The cytotoxicity of TSA was determined using the MTT assay test, while klf4 gene expression was assessed by real time PCR and to ability of TSA to induce apoptosis using a Vybrant Apoptosis Assay kit. **Results:** Our results showed that TSA exerted dose and time dependent cytotoxicity effect on SKOV-3 and A549 cells. Moreover TSA up-regulated klf4 expression. Flow cytometric analysis demonstrated that apoptosis was increased after TSA treatment. **Conclusions:** Taken together, this study showed that TSA increased klf4 expression in SKOV3 and A549 cell lines, consequently, klf4 may played a tumor-suppressor role by increasing both cell growth inhibition and apoptosis. This study sheds light on the details of molecular mechanisms of HDACi-induced cell cycle arrest and apoptosis.

Keywords: Histone deacetylase inhibitor - Trichostatin A - klf4 - apoptosis

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Introduction

Histone deacetylase inhibitors (HDACi) have emerged as a new class of targeted anticancer agents which mediates the regulation of gene expression and induces growth inhibition, apoptosis, differentiation and angiogenesis in a various of tumor cell lines (Glaser, 2007; Platta et al., 2007; Rasheed et al., 2007; Wu et al., 2007; Sonnemann et al., 2008; Bellarosa et al., 2012; Francisco et al., 2012; Shan et al., 2012; Feng et al., 2013). Owing to the high activity of HDACs in some types of cancers and reversible histone acetylation activity, HDAC has been recognized as excellent drug target for cancer therapy (Wdca, 2000; Weichert et al., 2008).

There are several types of HDACi, including SAHA, butyrate and trichostatin A (TSA). Hydroxamic acid HDAC inhibitor TSA is a compound with antifungal activity that was initially isolated from *Streptomyces hygroscopicus* (Owen et al., 1998). TSA is a specific and strong HDA-inhibitor (HDACi) which can alter gene expression and induce apoptosis in a variety of cancer

cells at very low concentration. The HDACi have been documented to increase the expression of pro-apoptotic proteins and caspase and repress the expression of anti-apoptotic Proteins (Sawa et al., 2001; Chen et al., 2002; Liu et al., 2006; Wilson et al., 2011; Horing et al., 2013; Zhang et al., 2013; Peiffer et al., 2014). The activity of a wide variety of non-histone transcription factors altered by acetylation, and affected by HDACi (Van et al., 1996; Glaser et al., 2003).

Several transcription factors such as NF- κ B, P53, E2F, Myc and klf4 are altered in human cancers (Mees et al., 2009). Krüppel-like factor 4 (klf4), formerly identified as a gut enriched KLF (GKLF) or epithelial zinc finger (EZF), is a zinc finger-containing transcription factor of the SP/KLF family, that expressed in a variety of tissues and regulates a range of biological processes, including cell proliferation, differentiation, tumorigenesis, apoptosis, and embryogenesis (Shields et al., 1996; Rowland et al., 2005; Wei et al., 2005; Wang et al., 2010; Aksoy et al., 2014). As a TF klf4 activates and represses the transcription of several genes that are involved in cell-cycle regulation

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and proliferation depending on the cellular context (Chen et al., 2003). klf4 activates the promoters of the negative cell-cycle-regulatory cyclin-dependent kinase inhibitor p21WAF1/Cip1 and p27Kip1 genes (Zhang et al., 2000; Daoyan et al., 2008). The studies Described that klf4 and p53 synergistically activate the p21WAF1/Cip1 promoter and are bound concurrently to the promoter of p21WAF1/Cip1 (Zhang et al., 2000). In addition, klf4 represses the promoters of several cell cycle-promoting genes such as CCND1 (cyclin D1) and CCNB1 (cyclin B1) (Shie et al., 2000; Yoon and Yang, 2004). These lead to cell cycle arrest at the G1/S phase and is also necessary to prevent the G2/M progression after DNA damage (Yoon et al., 2003; Yoon and Yang, 2004). As previously discussed, klf4 implicated in the regulation of apoptosis (Chen et al., 2000; hnishi et al., 2003). klf4 binds to the promoter of survivin gene, which is necessary for caspase inactivation and repress this promoter (Glaser et al., 2003).

Several lines of evidence indicated that klf4 either function as a tumor suppressor or an oncogen depending on genetic context of tumors. klf4 was primary recognized as a tumor suppressor in a variety of malignancies such as gastric, colon, ovarian, and lung cancers and its inactivation may play an essential role in tumor progression. Following studies have suggested an oncogenic role for klf4 in other cancers, including breast and dysplastic oral squamous epithelium (Rowland et al., 2005; Yori et al., 2011; Yu et al., 2011).

Up to our knowledge, the effect of TSA on klf4 expression was not studied previously. Also, regarding the importance of TFs in control of cancer progression, drug-induced apoptosis, and cell fate, our study was directed for elucidating the effect of HDACi TSA on proliferation and apoptosis of ovarian and lung cells. In addition we examined the possibility that klf4 may be a target gene for HDACi TSA and probable involvement of it in the inhibition of tumor proliferation. The findings of current paper may help elucidating mechanism of action of TSA in ovarian and lung cancer cell lines.

Materials and Methods

Cell culture

Ovarian and Lung cancer epithelial-like cell line, SKOV-3 and A549, were obtained from Pasteur Institute cell bank of Iran (code: C209, C137). These cells were cultured in RPMI 1640 medium (Gibco, Invitrogen, UK) supplemented with 10% fetal bovine serum (FBS (Gibco, Invitrogen, UK)), 100 µg/mL streptomycin (Merck co, Germany), and 100 U/ml penicillin (Serva co, Germany), and 0.37% (w/v) NaHCO₃. Cells were incubated at 37°C in 5% CO₂ and 95% humidified air. Media were changed twice weekly (45-64).

In vitro cytotoxicity

The cytotoxicity of TSA (Sigma-Aldrich) was determined using the MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide) assay (MTT; Sigma-Aldrich), which is based on the conversion of the yellow tetrazolium salt MTT to purple formazan crystals by mitochondrial dehydrogenases. In brief, Viable cells were counted using trypan blue (0.02%) and seeded in 96-well

Table 1. Sequences of Primers for RT-PCR

Primer name	Primer length	Sequence (5' to 3')	Product size (bp)
klf4 forward	20	GGCACCTACCGTAAACACACG	40
klf4 reverse	20	CTGGCAGTGTGGGTCATATC	40
β-actin forward	20	TCCCTGGAGAAGAGCTACG	131
β-actin reverse	20	GTAGTTTCGTGGATGCCACA	131

plates at a density of 3×10³ (SKOV-3) and 2×10² (A549) cells/well and allowed to attach and equilibrate for 24 h in a humidified atmosphere of 95% air and 5% CO₂. They then treated with various concentrations of TSA for 24, 48 and 72 h. After the end of incubation, 10 µl MTT (5 mg/ml PBS) was added to each well for 4 h the supernatant was removed and 200 µl dimethyl sulfoxide (DMSO) were added to dissolve the formazan salt formed. Absorbance was measured at 570 nm using an ELISA Reader.

RNA extraction and cDNA synthesis

Cells were treated with TSA (100 nM) for 72 h. Total RNA from non-treated and TSA-treated cells was extracted using RNX-Plus solution (CinaGen, Iran) according to the manufacturer's instructions. Total RNA was quantified using a photospectrometer (NanoDrop nd-1000 uv/vis, U.S.A.) and RNA integrity was confirmed by electrophoresis of samples on a 1.2% agarose gel. The cDNA was synthesized from 1 µg of total RNA from each sample, and 1 µl of random hexamer primers (Roche Applied Science) was diluted to a final volume of 20 µl in DEPC-treated water using a AccuPower RocketScript RT PreMix kit (BIONEER, Korea) according to the manufacturer's protocol. cDNA synthesis was performed under the following condition, annealing at 15°C for 1 min, cDNA synthesis 50°C for 1h and Heat inactivation 95°C for 5 min. The samples can be stored at -20°C until use.

Quantitative Real-time PCR

Real-time PCR was performed in a Corbett (Rortor Gene 6000) system using AccuPower 2X Greenstar qPCR Master Mix (BIONEER, Korea). β-Actin was used as a housekeeping gene for standardization of klf4 mRNA expression. The primer sequences used in our study were showed in Table 1.

A total of 20 µl of PCR reaction mixture was made using 2 µl cDNA, 1 µl forward primer, 1 µl reverse primer, 10µl SYBR premix EX Taq, and 6µl of DEPC-treated water. The thermal profile for real-time PCR was 95°C, 10 minutes, 1 cycle (Holding step); 95°C, 15 seconds, 58°C 45 second 40 cycles (cycling step); 72-95°C, 1 cycle (Melting step). Expression of each amplicon was analyzed by using the 2^{-ΔΔCt} method, where ΔΔCt is the ΔCt (klf4) - ΔCt (actin) and Ct is the cycle at which the threshold is crossed.

Flow cytometry analysis for apoptosis

The ability of TSA to induce apoptosis of SKOV-3 and A549 cells was evaluated using the Vybrant Apoptosis Assay kit (Invitrogen, USA), according to the manufacturer's Protocol. This assay was based on the differential permeability of the apoptotic to the green fluorescent dye (YO-PRO-1) and necrotic cells to the red fluorescent dye [propidium iodide (PI)]. YO-PRO-1 can

enter the apoptotic cells and bind to DNA or RNA, which significantly enhances its fluorescence intensity. cells were cultured in 6 well plates for 72 h and then treated with various concentration of TSA. After 72 h treatment, Approximately 5×10^5 Cells collected by trypsinization and washed and re-suspended in 1 ml of phosphate-buffered saline (PBS). YO-PRO-1 and PI was added at a final concentration of each prob 1 μ l then incubated for 30 min in the dark at 4°C. Cells were analyzed by FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with flowjo software and the percentages of apoptotic (YO-PRO-1+/PI and necrosis (YO-PRO-1/PI+) cells were determined.

Statistical analysis

Results are presented as mean and standard error of the mean (SEM). Data were analyzed using ANOVA and Tukey's test to calculate the significance values; a p-value < 0.05 was considered as significant. All experiments were replicated at least three times to confirm the result.

Results

Inhibitory effects of TSA on SKOV-3 and A549 cells proliferation

The MTT assay was used to evaluate viability of SKOV-3 and A549 cells exposed to different concentrations of TSA for 24, 48 and 72 h. The result in SKOV-3 and A549 cells showed that the survival rate of treated cells decreased with the increasing concentration of TSA (see Figure 1), which was also dependent on the time of incubation. The IC_{50} parameters determined for TSA were 5.6, 0.7, 0.32 μ M in SKOV-3 cells and 3.2, 0.28, 0.06 μ M in A549 cells after 24, 48, and 72h treatments,

respectively.

TSA Stimulates *klf4* Expression in SKOV-3 and A549 cells

To examine TSA effects in *klf4* mRNA expression in human Ovarian, Lung cell lines, we analyzed the level of *klf4* mRNA by real time RT-PCR in SKOV-3 and A549 cell lines in the presence and absence of TSA. Changes in *klf4* mRNA expression levels between the treated and Control cells were normalized to beta-actin mRNA levels and calculated by the $2^{-\Delta\Delta Ct}$ formula. The results demonstrated that treatment with TSA induced an increase in *klf4* mRNA levels in these cell lines. The induction of *klf4* mRNA expression by TSA was reverse dose dependent. A 9.3 and 15.5-fold increase occurred in SKOV-3 cells treated with 0.32 and 0.22 μ M TSA and 2.1 and 8.57 fold increase occurred in A549 cells treated with 0.06 and 0.04 TSA concentration.

Induction of apoptosis by TSA in SKOV-3 and A549 cells

Apoptosis in SKOV-3 and A549 cells was assessed by flow cytometry. Flow cytometry was used to distinguish the uptake of YO-PRO-1 by apoptotic cells. YO-PRO-1 is a green fluorescent dye, which demonstrate considerable fluorescence enhancements upon binding to nucleic acids. While propidium iodide (PI), red fluorescence dye, is applied to stain necrotic cells. As shown in Figure 2 and 3, in SKOV-3 cells the percentage of apoptotic cells in 0.42, 0.32, and 0.22 μ M concentration was 8.58, 15.3, and 19.5% and in A549 cells in 0.08, 0.06, and 0.04 μ M concentration was 14, 18.7, and 25.8%, respectively after 72 h of incubation. We used different concentration of TSA according to the results of MTT assay. The concentration of TSA are around IC_{50} ranges. According to the results the number of apoptotic cells labeled with YO-PRO-1

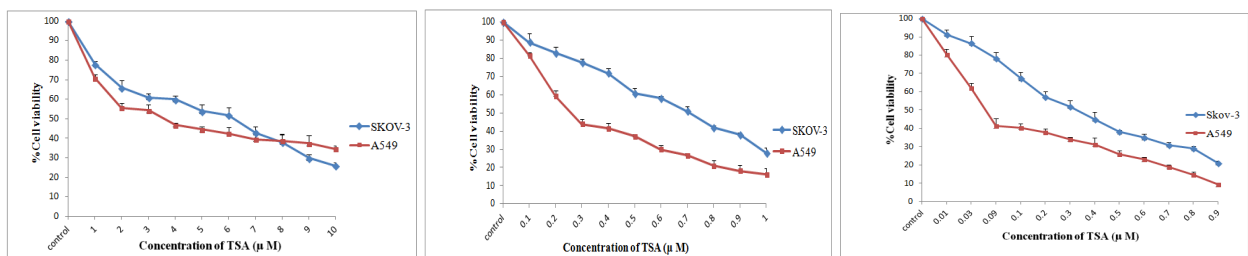


Figure 1. A: Effect of TSA on Survival of Skov-3 and A549 Cells. Cells were treated with different concentrations of TSA for 24(A), 48(B), and 72(C) h. Cell viability was measured by MTT assay

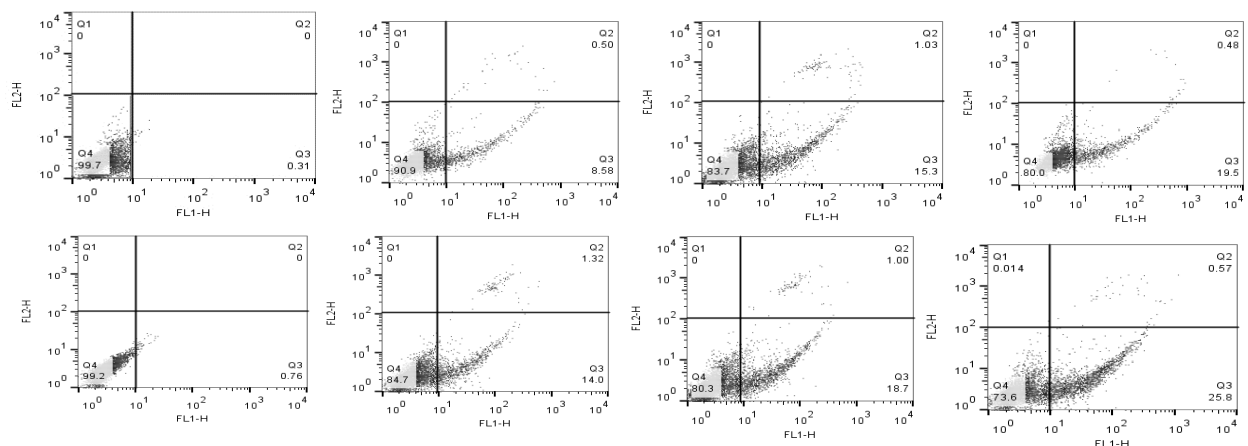


Figure 2. Apoptosis in (a) Skov-3 and (b) A549 Cells was Assessed by Flow Cytometry

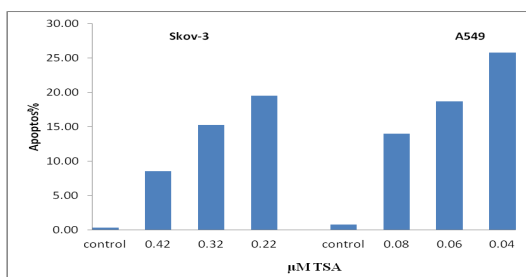


Figure 3. Effect of TSA on Apoptosis of Skov-3 and A549 Cells. After cultured with various concentrations of TSA for 72 h, cells were stained with yo-pro/propidium iodide, and analyzed by flow cytometry

increased with decreasing TSA concentration.

Discussion

The aim of this study was to determine the response of the cell lines: SKOV-3 and A549 from Ovarian and Lung cancers, respectively to treatment with the histone deacetylase inhibitor TSA. Previous studies have shown that Histone deacetylase inhibitors have antitumor activity in a variety of cancers and compared with conventional chemotherapeutic drugs, Histone deacetylase inhibitors have tumor selectivity and less toxicity in normal cells (Butler et al., 2000; Butler et al., 2001; Vigushin et al., 2001; Cheng et al., 2012; Zhang et al., 2012; Wang et al., 2013). Recent studies have demonstrated that TSA induces cell growth inhibition, cell-cycle arrest and apoptosis in hela, bladder, pancreatic, Glioblastoma, breast, and colon cancer cell lines through Zn ion chelating (Paul et al., 2000; Sawa et al., 2001; Vigushin et al., 2001; Paolo et al., 2006; Horing et al., 2013; You and Park, 2013). Epigenetical interference of HDACis is now thought to play important roles to avert onset and progression of cancer in several tumor types.

There are only a few studies examining TSA effects in human ovarian and lung cancer cell lines. We investigated the effect of TSA on the cell viability and apoptosis of human epithelial ovarian and lung carcinoma cell lines (SKOV-3 and A549 cells). Our current findings indicate that the induction of cell death by TSA was dose and time dependent and occurred at a very low concentration. In accordance with this study Guang et al showed that TSA have cytotoxicity effect in dose dependent manner in breast cancer cells and the nanomolar concentrations of TSA are effective (Guang et al., 2012).

This study showed that TSA could induce apoptosis in both cell lines SKOV-3 and A549, but the rate of apoptosis in A549 cells was more than SKOV-3. The possible reason is type of P53 gene, mutant in SKOV-3 and wild type in A549. In accordance with this data, Z.Song et al indicated that TSA induced apoptosis 17.6% after 24h of treatment (38). C. Herold et al illustrated time-dependent increase of apoptosis in the hepatoma cell lines (18 and 32% for HepG2 vs 38 and 50% for Hep1B after 24 and 72 h TSA treatment) (Herold et al., 2002).

The anti-neoplastic properties of HDAC inhibitors are mediated by their ability to induce histone hyperacetylation, resulting in the altered transcription of key genes, some of them involving in regulation of cell proliferation and

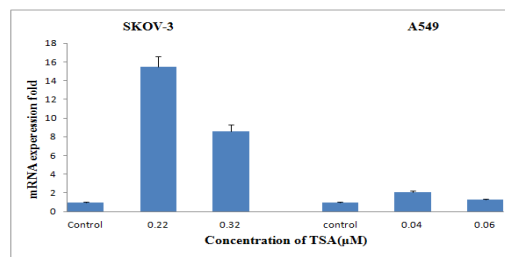


Figure 4. Effect of TSA on KLF4 Gene Expression in Skov-3 and A549 Cells. Relative expression levels of KLF4 mRNA at various concentration points as indicated as determined by real-time PCR. The amount of KLF4 mRNA was normalized to an endogenous reference, β -actin.

apoptosis (Choudhary et al., 2009).

This study is the first to demonstrate trichostatin A effects on klf4 mRNA expression in ovarian and lung cancer cells. In present study, we exhibited TSA considerably induced the expression of tumor suppressor gene klf4 on mRNA level in human ovarian and lung cancer cell lines. Several studies indicated that klf4 has variable effects on cell cycle arrest and induction of apoptosis depending on the genetic context of tumors (as a tumor suppressor or an oncogene). The work of Zhi Yi Chen et al that they showed TSA and butyrate upregulated klf4 mRNA levels in HT-29 cells, We achieved similar data in SKOV-3 and A549 cells. On the other hand, some papers have revealed that klf4 mRNA expression was significantly decreased in ovarian and lung cancers (Rowland et al., 2005; Choia et al., 2006; Yori et al., 2011; Yu et al., 2011; Yoon and Roh, 2012). The down regulation of klf4 leads to uncontrolled cell proliferation and tumor development. Other reports have also shown that klf4 can repress HDAC gene expression (Ai et al., 2004). However molecular mechanisms of HDACi-induced cell cycle arrest and apoptosis, and the regulation of gene expression remain unclear and Future studies about is needed.

In conclusion, the results of the present study indicate that trichostatin A have inhibitory effect in ovarian and lung cancer cell lines (SKOV-3 and A549). This inhibition was dose and time dependent. The result of flow cytometry show trichostatin A induce apoptosis in dose dependent manner. Increased klf4 expression in SKOV3 and A549 cell lines, consequently, klf4 may played a tumor-suppressor role by increasing both the cell growth inhibition and appoptosis. Also, we emphasized the possibility of klf4 as a downstream target of TSA. This study can shed light on the details of molecular mechanisms of HDACi-induced cell cycle arrest and apoptosis.

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