MEDICAL SCIENCE MONITOR

MOLECULAR BIOLOGY

e-ISSN 1643-3750
© Med Sci Monit, 2017; 23: 5793-5802
DOI: 10.12659/MSM.904597

Received: 2017.03.30
Accepted: 2017.05.19
Published: 2017.12.06

Histone Deacetylase (HDAC) Inhibitor, Suberoylanilide Hydroxamic Acid (SAHA), Induces Apoptosis in Prostate Cancer Cell Lines via the Akt/FOXO3a Signaling Pathway

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Data Interpretation D
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Source of support: Departmental sources

Background: Histone deacetylase (HDAC) inhibitors are emerging as a new class of anti-cancer drugs that promote cancer cell apoptosis, and include suberoylanilide hydroxamic acid (SAHA). The aim of this study was to investigate the mechanism of SAHA-induced apoptosis in human prostate cancer cell lines, DU145 and PC-3.

Material/Methods: Cell lines, DU145 and PC-3, were studied before and after treatment with SAHA. The effects of SAHA treatment on cell proliferation were studied using the MTT cell proliferation assay. Annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining were used to study the effects of SAHA treatment on cell apoptosis. Western blotting, quantitative polymerase chain reaction (qPCR) and short interfering (si)RNA assays were performed to study the effects of SAHA treatment on apoptotic and cell cycle proteins and the Akt/FOXO3a signaling pathway.

Results: Treatment with SAHA inhibited cell proliferation in human prostate cancer cell lines DU145 and PC-3 cells in a dose-dependent way. Cell cycle analysis and Annexin-V FITC/PI staining showed that treatment with SAHA resulted in G2/M cell cycle arrest and increased cell apoptosis in a dose-dependent way. Also, treatment with SAHA reduced the protein expression levels cyclin B and cyclin A2 and promoted the activation of FOXO3a by inhibiting Akt activation. Western blotting, the siRNA assay, and qPCR showed that FOXO3a, the Bcl-2 family of proteins, survivin, and FasL were involved in SAHA-induced apoptosis in prostate cancer cells grown *in vitro*.

Conclusions: Treatment with SAHA promoted apoptosis via the Akt/FOXO3a signaling pathway in prostate cancer cells *in vitro*.

MeSH Keywords: Apoptosis • Cell Cycle Checkpoints • Prostatic Neoplasms

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/904597

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Background

Prostate cancer is the second most frequently diagnosed cancer in men worldwide, and the second leading cause of cancer-related death [1]. Prostate cancer accounts for 21% of all new cancer diagnoses in men in the United States, and it commonly presents with metastases [1]. The androgen receptor (AR) plays a crucial role in prostate tumor growth. Therefore, the traditional treatment of advanced prostate cancer includes androgen ablation or the use of drugs that target AR signaling [2]. Androgen-deprivation therapy (ADT) is effective initially, but the therapeutic benefits are of short duration, and advanced prostate cancer may develop into castration-resistant prostate cancer (CRPC) within 18–24 months [3]. Currently, taxane-based chemotherapy is the most common treatment for the patients with metastatic CRPC [4]. Docetaxel plus prednisone was approved as first-line chemotherapy for CRPC in 2004, but most patients with docetaxel treatment experienced progression of CRPC within one year [5], with death occurring within three years of diagnosis [6]. These clinical findings suggest that CRPC remains poorly managed and new medical therapies are urgently needed.

Drug resistance in metastatic CRPC could be due to a variety of causes, including activation anti-apoptotic pathways and proliferation pathways and crosstalk between the AR and these pathways [5]. The use of combination treatment of two or more chemotherapeutic drugs could reduce drug resistance in prostate cancer. For example, the use of histone deacetylase (HDAC) inhibitors in combination with other chemotherapeutic agents or radiotherapy have shown promising results [7,8]. It is possible that the combined use of taxanes and HDAC inhibitors might exhibit improved responses in CRPC. Therefore, further studies to investigate the mechanism of HDAC inhibitors in prostate cancer are warranted.

HDAC inhibitors are a new class of anti-tumor drugs that modify the epigenetic profile and alter gene expression in malignant cells. HDAC inhibitors are classified into four classes that include: short chain fatty acids, hydroxamic acids, cyclic peptides, and synthetic benzamides [9]. HDAC inhibitors can cause cell cycle arrest, induce apoptosis, generate reactive oxygen species (ROS), and inhibit DNA repair by promoting acetylation of histones and/or non-histone protein substrates [9]. There is now accumulating published evidence to indicate that HDAC inhibitors, including depsipeptide (FK228), LAQ824, and suberoylanilide hydroxamic acid (SAHA) are a promising class of anti-cancer agents in the treatment of solid and hematological malignancies [9].

HDAC inhibitors induce apoptosis by the extrinsic cell apoptosis pathway or the intrinsic mitochondrial pathway [10]. Several HDAC inhibitors have been shown to promote apoptosis by regulating the expression of FasL [11], Bim, Bcl-2 or Bax in human leukemia cells [12]. Forkhead box O3a (FOXO3a) can be phosphorylated by Akt, and promotes cell apoptosis by either inducing expression of Bcl-2 family or upregulating the cell death receptor ligand FasL [13]. It is reasonable to speculate that the Akt/FOXO3a pathway might be involved in HDAC inhibitor-induced cell apoptosis.

SAHA (Vorinostat) is a pan-HDAC inhibitor that structurally belongs to the group of hydroxamic acids. SAHA (Vorinostat) was the first FDA-approved HDAC inhibitor and is clinically effective in the treatment of refractory primary cutaneous T-cell lymphoma [14]. SAHA has been shown to be an effective inhibitor of tumor cell growth in prostate cancer [15]. Recent studies have demonstrated that combined treatment with SAHA and other chemotherapeutic drugs, such as the pan-aurora kinase inhibitor, AMG 900, showed synergistic anti-cancer activity in prostate cancer [16,17]. Different signaling pathways are involved in SAHA-induced apoptosis, including the caspase-dependent apoptotic pathway, signal transducer and activator of transcription (STAT), extracellular signal-regulated kinases (ERK), and the mechanistic target of rapamycin (mTOR) pathways [18, 19]. HDAC inhibitors are emerging as a new class of anti-cancer drugs that induce cancer cell apoptosis and include SAHA. The aim of this study was to investigate the mechanism of SAHA-induced apoptosis in two human prostate cancer cell lines, DU145 and PC-3.

Material and Methods

Cell culture and treatment

Human prostate cancer cell lines DU145 and PC-3 cells were obtained from Cell Bank of Type Culture Collection, Chinese Academy of Science (Shanghai, China). Cells were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS). Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C. Cells were treated with different concentrations of the histone deacetylase (HDAC) inhibitor, suberoylanilide hydroxamic acid (SAHA) (Sigma-Aldrich, St Louis, MO, USA). DU145 cells were treated with 0, 1, 2, 4, 8, 16, 32 μM SAHA, and PC-3 cells were treated with 0, 0.25, 0.5, 1, 5, 10, 15 μM SAHA, respectively.

The MTT cell proliferation assay

DU145 and PC-3 cells were seeded in 96-well plates and incubated for 24 h prior to SAHA treatment. Cells were treated with different doses of SAHA for 24 h and 48 h. Then, 20 μl MTT was added to each well and incubated for 4 h at 37°C. Culture medium was removed and MTT formazan crystals were dissolved in 150 μl dimethylsulfoxide (DMSO) (Sigma-Aldrich). Absorbance was measured at a wavelength of 570 nm and

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background absorbance was subtracted (at 690 nm) with the use of a microplate reader (Model 680, Bio-Rad Laboratories, Hercules, CA, USA). All procedures were repeated in triplicate.

Cell cycle analysis

A cell cycle analysis kit (Beyotime, Haimen, China) was used, according to the manufacturer's instructions. DU145 and PC-3 cells were seeded in 6-well plates. After treatment, cells were washed twice with cold phosphate-buffered saline (PBS), and then fixed in 70% ethanol in PBS at –20°C for 24 h. After fixation, cells were washed in cold PBS and then stained with propidium iodide (PI) at 37°C for 30 min in the dark. The samples were analyzed by flow cytometry (BD Biosciences, San Jose, CA, USA). All experiments were performed in triplicate.

Annexin-V-FITC/PI staining

Annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining were performed using an Annexin-V-FITC/PI kit (Keygen, Biotech, Nanjing, China) according to the manufacturer's instructions. Briefly, DU145 and PC-3 cells were cultured in 6-well plates and treated with different doses of SAHA for 48 h. Cells were then harvested using 0.05% trypsin and washed twice with cold PBS, and then resuspended in binding buffer. Then, 1×10^5 cells in 100 µl binding buffer were added to a 5 ml tube, and 5 µl of Annexin-V-FITC reagent and 5 µl of PI were added to each tube. Cells were gently mixed and incubated for 15 min at room temperature. 400 µl binding buffer was then add to each tube. The samples were analyzed by flow cytometry (BD Biosciences). All experiments were performed in triplicate.

Western blotting

Protein extracts from DU145 and PC-3 cells were prepared in Mammalian Cell Lysis/Extraction Reagent (Sigma-Aldrich). The protein concentration was determined using the Bradford reagent (Bio-Rad). Equal amounts of protein lysate were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane (Bio-Rad). Membranes were blocked with 5% non-fat milk powder in PBS-Tris buffer for 1 h, followed by incubation with primary antibody at 4°C overnight. Membranes were then incubated with the corresponding secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h. Enhanced chemiluminescence Western blotting detection reagents (GE Healthcare, Piscataway, NJ, USA) were used for protein detection. The X-ray films were scanned and bands were analyzed.

RNA isolation and quantitative polymerase chain reaction (qPCR)

Total RNA was isolated from DU145 and PC-3 cells using Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and reverse transcribed into cDNA using a PrimeScript RT reagent kit with cDNA Eraser (TaKaRa, Dalian, China). Reverse transcription (RT) products were used as templates for subsequent qPCR.

The following primers were used in this study:
Primers for *FasL* were:
forward 5'-GAAGAGAGGGAACCACAGCA-3',
reverse 5'-TTGCCTGTTAAATGGGCCAC-3'.
Primers for *Bim* were:
forward 5'-TCATCGCGGTATTCGGTTCG-3',
reverse 5'-CTTCACCTCCGTGATTGCCT-3'.
Primers for *GAPDH* were:
forward 5'-GTCAGTGGTGGACCTGACCT-3',
reverse 5'-TGGTGCTCAGTTTAGCCCAGG-3'.

The mRNA levels of the target genes were analyzed by the ABI7900 Real-Time PCR Detection System (Applied Biosystems, Foster City, CA, USA) with Syber Green reagent (Thermo Fisher Scientific). GAPDH was used as an internal control for normalization. The specificity of the fluorescence signal was confirmed by both melting curve analysis and agarose gel electrophoresis. The mRNA levels of target genes were determined by the $2^{-\Delta\Delta Ct}$ method.

Knockdown of FOXO3a by RNAi in DU145 and PC-3 cells

DU145 and PC-3 cells were cultured for 24 h prior to transfection. These cells were then transfected with non-targeting control short interfering (si)RNA (ID# 4390843) or pre-designed Silencer Select siRNA for human FOXO3a (ID# 115209, Thermo Fisher Scientific) using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific) according to the manufacturer's instruction. At 48h post-transfection, cells were treated with SAHA for 48 h.

Statistical analysis

All experiments were performed in triplicate. Data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed by one-way ANOVA. In selected experiments, a Student's t-test was used for paired comparisons. Statistical analysis was performed using the SPSS 17.0 for Windows software (SPSS Inc., Chicago, IL, USA). A P-value <0.05 was considered to be statistically significant.

Results

SAHA treatment resulted in dose-dependent inhibition of cell proliferation of DU145 and PC-3 cells

To explore the anti-tumor activity of the histone deacetylase (HDAC) inhibitor, suberoylanilide hydroxamic acid (SAHA) in

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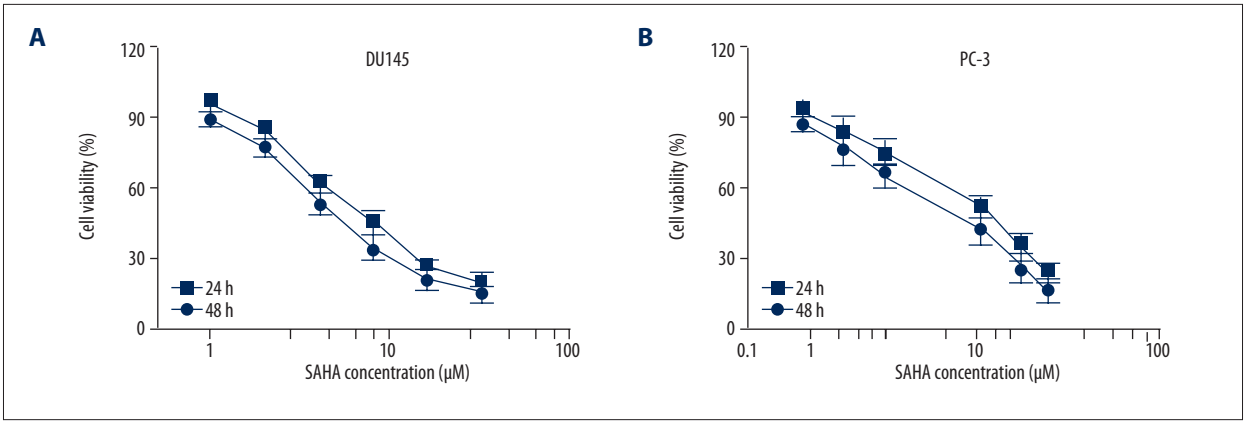

Figure 1. Effect of suberoylanilide hydroxamic acid (SAHA) on cell proliferation in DU145 and PC-3 cells. (**A**) DU145 cells were treated with different doses of SAHA (0, 1, 2, 4, 8, 16, 32 μM) for 24 and 48 h. (**B**) PC-3 cells were treated with different doses of SAHA (0, 0.25, 0.5, 1, 5, 10, 15 μM) for 24 and 48 h. Cell viability was monitored by the MTT cell proliferation assay. SAHA concentration (μM) after $\log_{10}$ transformation is represented on the X-axis. Data were presented as the mean ± standard deviation (SD) and performed in triplicate.

prostate cancer cells, human prostate cancer cell lines DU145 and PC-3 cells were treated with increasing doses of SAHA for 24 and 48 h. The MTT cell proliferation assay was performed to monitor the cell proliferation. As shown in Figure 1A and 1B, SAHA inhibited cell proliferation of DU145 and PC-3 cells in a dose-dependent manner, whereas the extension of the incubation time to 48 h did not significantly enhance the sensitivity of cells to SAHA. The cell viability of DU145 cells was decreased by about 55% upon SAHA (4 μM) treatment for 48 h, and the viability of PC-3 cells was reduced to about 45% in the presence of 5μM SAHA. According to the IC50 values, which were calculated based on the MTT cell proliferation assay, three different doses of SAHA were selected for the subsequent experiment. The selected doses of SAHA for DU145 cells were 1, 3, 9 μM; 0.5, 2, 8 μM SAHA were selected for the treatment of PC-3 cells. The treatment time for the subsequent studies was 48 h.

SAHA treatment resulted in dose-dependent G_2/M cell cycle arrest in DU145 and PC-3 cells

To characterize whether SAHA caused DU145 and PC-3 cells to arrest in a specific cell cycle phase, these cells were treated with different doses of SAHA for 48 h. Propidium iodide (PI) staining and flow cytometry were further performed to show the cell cycle distribution of the cells. The highest doses of SAHA increased the percentage of cells in G_2/M phase to about 30% in both DU145 and PC-3 cells (Figure 2A, 2B). SAHA treatment resulted in G_2/M cell cycle arrest in both DU145 and PC-3 cells (Figure 2A, 2B). The percentage of cells in G_0/G_1 phases was decreased with a concomitant increase in cells in the sub-G_0/G_1 phases following SAHA treatment (Figure 2A, 2B, lower panel). The increase in the sub-G_0/G_1 population was indicative of cell death. These data suggest that SAHA induced G_2/M cell cycle arrest in DU145 and PC-3 cells in a dose-dependent way.

SAHA treatment induced dose-dependent cell apoptosis in DU145 and PC-3 cells

Since cell death was observed by the increase in the sub-G_0/G_1 populations, we next examined whether SAHA induced cell apoptosis in DU145 and PC-3 cells. Annexin-V-FITC/PI staining was performed. As shown in Figure 3A and 3B, SAHA induced apoptosis in both DU145 and PC-3 cells in a dose-dependent way. The highest doses of SAHA increased the apoptotic rate of DU145 and PC-3 cells to 18.44% and 26.71%, respectively (Figure 3A, 3B). The quantitative data of Annexin-V-FITC/PI staining are shown in Figure 3C.

SAHA treatment resulted in a dose-dependent decrease in the levels of cell cycle regulatory proteins, phosphorylated Akt and FOXO3a

To further investigate the signaling pathway involved in cell cycle arrest and apoptosis upon SAHA treatment, we detected the expression levels of cell cycle regulatory proteins and phosphorylation of Akt and FOXO3a in both DU145 and PC-3 cells. Previous studies have shown that HDAC inhibitors cause G_2/M arrest by regulating cyclin B and cyclin A2 in tumor cells [20,21], which is why these two proteins were selected in this study. As shown in Figure 4A, SAHA decreased the protein levels of cyclin B and cyclin A2 in DU145 and PC-3 cells. Also, phosphorylation of Akt and FOXO3a were detected as the putative apoptotic pathway following SAHA treatment. SAHA caused a reduction of p-Akt and p-FOXO3a (Figure 4A). These data suggest that SAHA resulted in cell cycle arrest and apoptosis via downregulating cyclin B, cyclin A2, p-Akt and p-FOXO3a in DU145 and PC-3 cells. The quantitative data of Western blotting are shown in Figure 4B and 4C.

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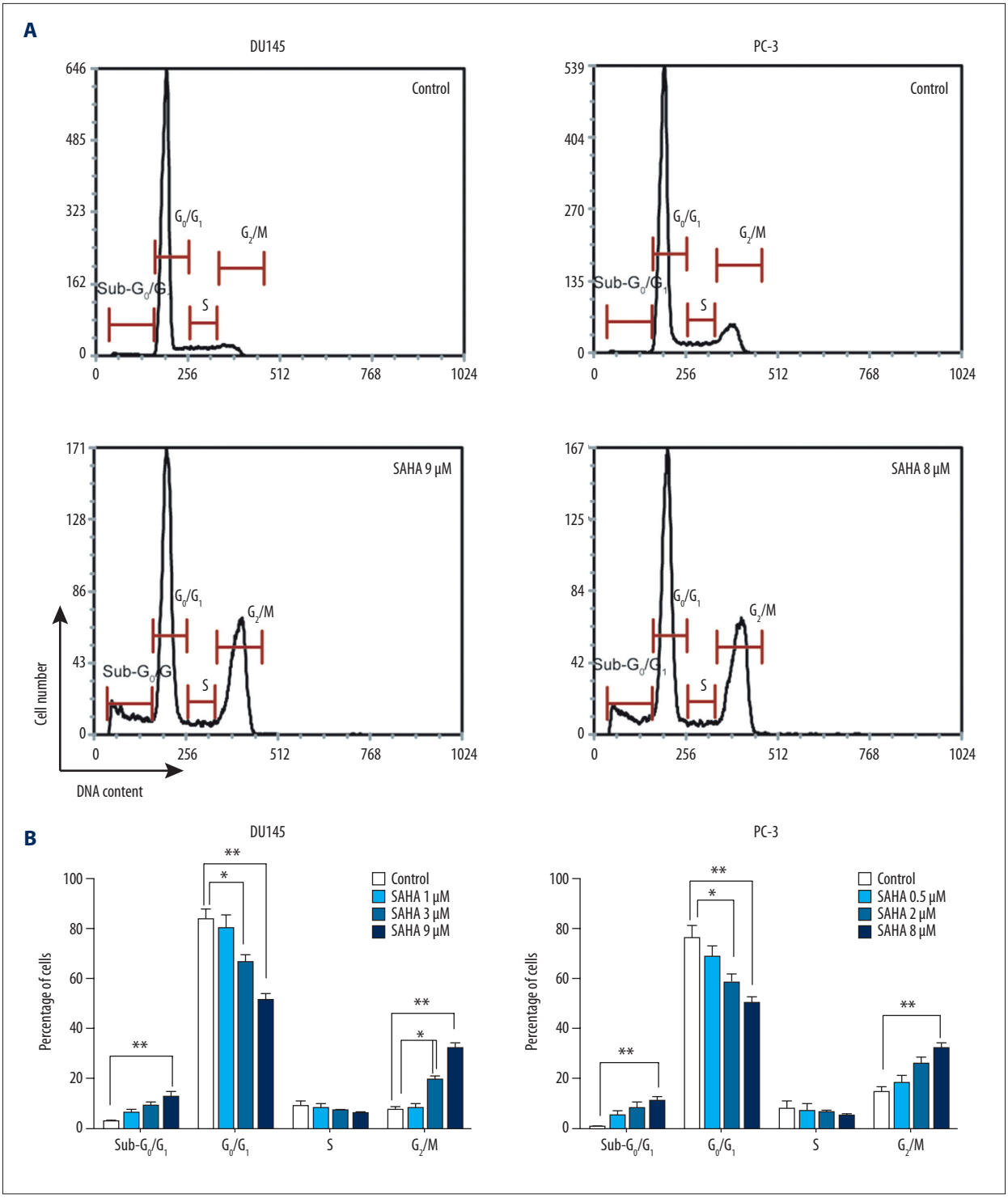

Figure 2. Effect of suberoylanilide hydroxamic acid (SAHA) on the cell cycle in DU145 and PC-3 cells. (**A**) DU145 cells were treated with four doses of SAHA (0, 1, 3, 9 μM) for 48 h. PC-3 cells were treated with four doses of SAHA (0, 0.5, 2, 8 μM) for 48 h. Cells were stained with propidium iodide (PI) and analyzed by flow cytometry. (**B**) Quantitative data of PI staining in DU145 and PC-3 cells. Data were presented as the mean ± standard deviation (SD) and performed in triplicate. * $P<0.05$,** $P<0.01$ versus the control group.

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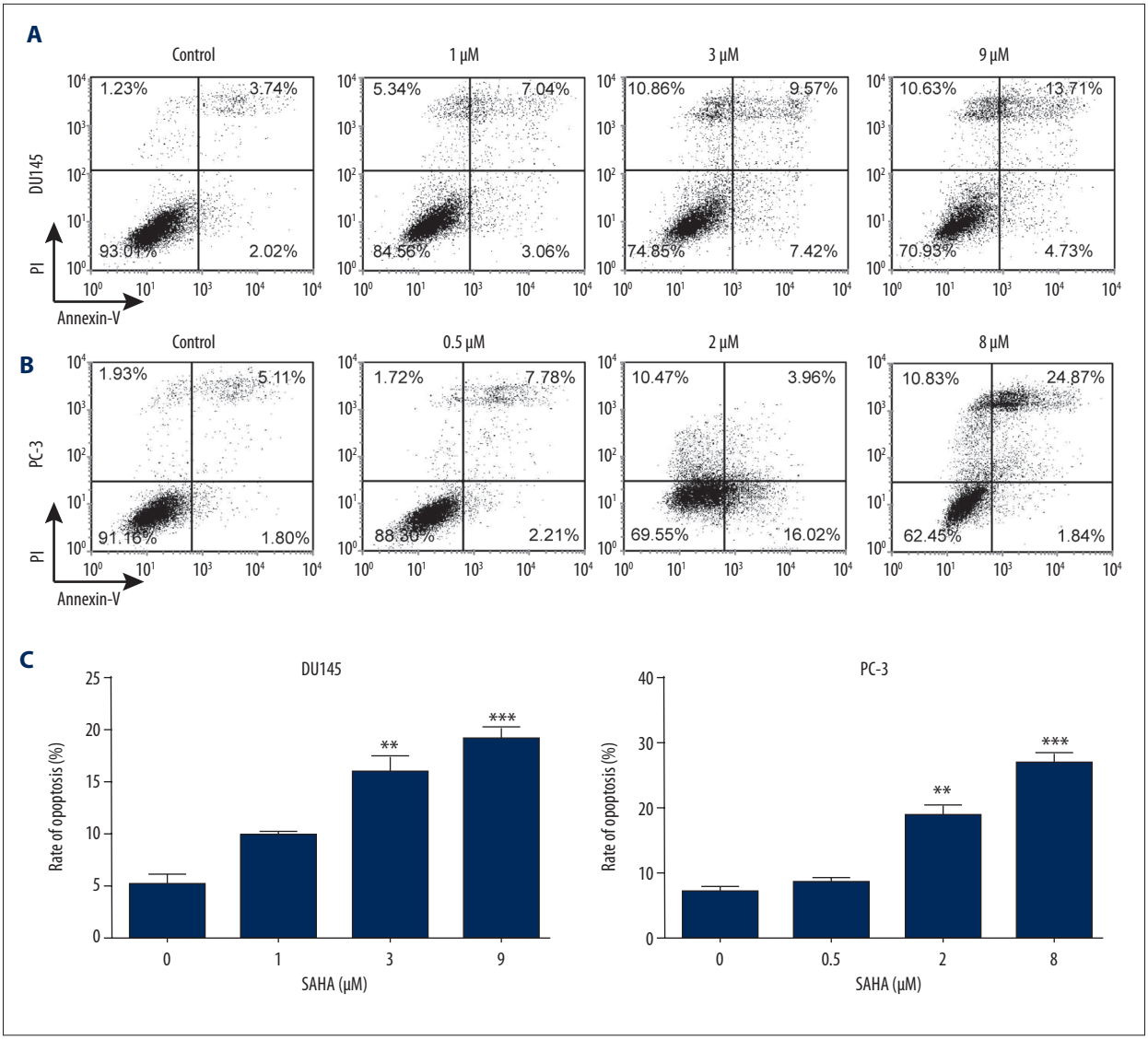

Figure 3. Effect of suberoylanilide hydroxamic acid (SAHA) on cell apoptosis in DU145 and PC-3 cells. (**A**) DU145 cells were treated with four doses of SAHA (0, 1, 3, 9 μM) for 48 h. (**B**) PC-3 cells were treated with four doses of SAHA (0, 0.5, 2, 8 μM) for 48 h. Cells were stained with Annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide (PI). The apoptotic rate was determined by flow cytometry. (**C**) Quantitative data of Annexin-V-FITC/PI staining. Data were presented as the mean ± standard deviation (SD) and performed in triplicate. ** $P<0.01$, *** $P<0.001$ versus the control group.

Identification of SAHA-induced apoptotic pathways in DU145 and PC-3 cells

In order to study the SAHA-induced apoptotic signaling pathways, we next examined the expression of apoptosis-related genes, including the Bcl-2 family proteins and survivin. The results of Western blotting showed that SAHA upregulated the expression of Bim and Bax (pro-apoptotic proteins) with concomitant decrease of Bcl-2 and survivin (anti-apoptotic proteins) in a dose-dependent manner (Figure 5A). In addition, the quantitative results indicated that the ratio of Bcl-2/Bax significantly decreased after SAHA treatment in both DU145 and PC-3 cells (Figure 5C). The results of qPCR further showed that the mRNA levels of Bim and FasL were increased by SAHA (Figure 5B). The mRNA induction of Bim was consistent with the results of Western blotting (Figure 5A). These findings suggest that Bcl-2 family and survivin are involved in SAHA-induced apoptosis in prostate cancer cells.

Knockdown of FOXO3a partially reduced the effect of SAHA treatment on apoptosis-related proteins

It has been previously established that FOXO3a can promote cell apoptosis by regulating expression of Bcl-2 family proteins,

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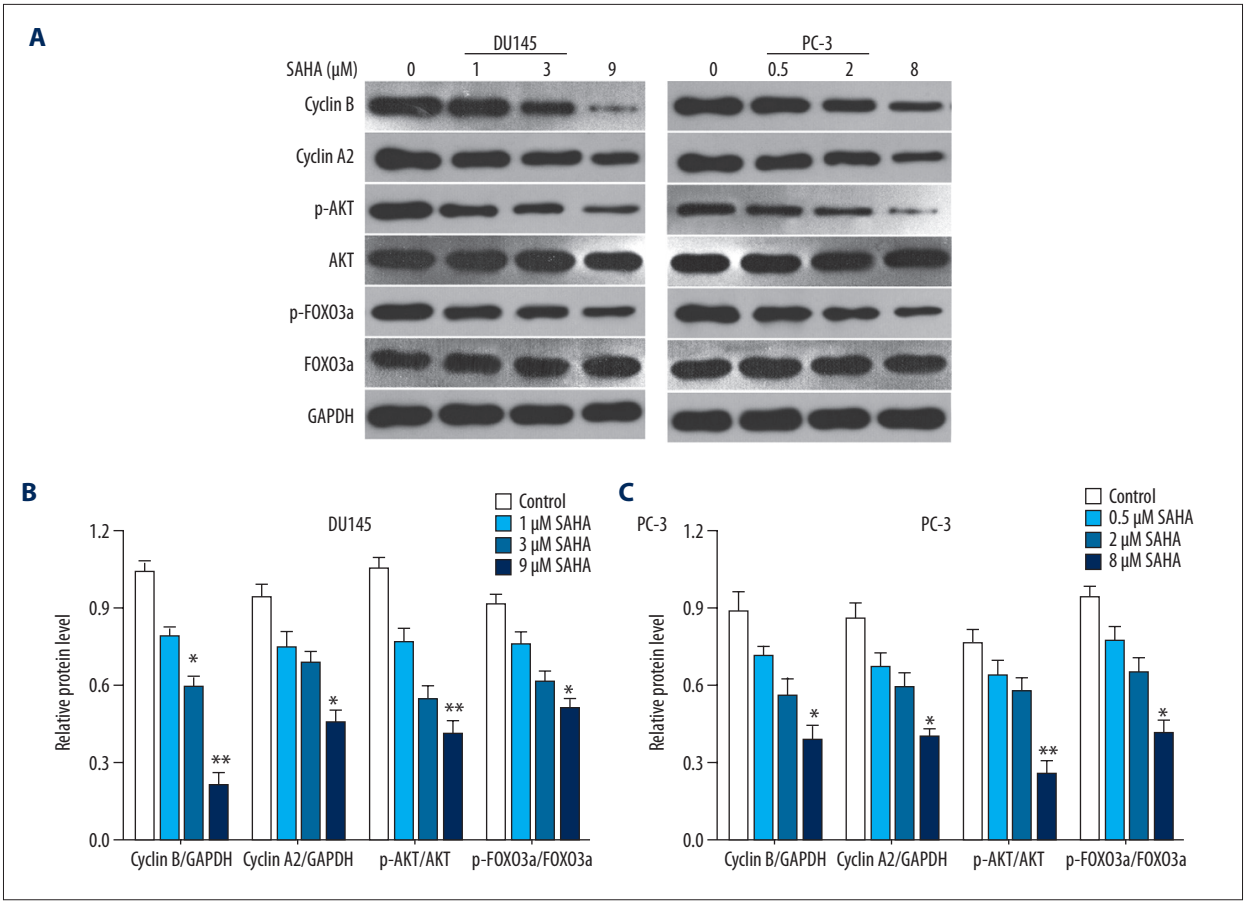

Figure 4. Western blotting analysis of cyclins and phosphorylation of Akt and FOXO3a in DU145 and PC-3 cells. (**A**) DU145 cells were treated with four doses of SAHA (0, 1, 3, 9 μM) for 48 h. PC-3 cells were treated with four doses of SAHA (0, 0.5, 2, 8 μM) for 48 h. Expression of cyclin A2, cyclin B, phosphorylated Akt/FOXO3a and total Akt/FOXO3a were analyzed by Western blotting. GAPDH served as a loading control. Data are representative images of three independent experiments. (**B, C**) Quantitative data of Western blotting in DU145 and PC-3 cells. Data were presented as the mean ± standard deviation (SD) and performed in triplicate. * $P<0.05$, ** $P<0.01$ versus the corresponding control.

modulating survivin expression or upregulating cell death receptor ligands FasL [13,21]. In order to investigate the role of FOXO3a in the SAHA-mediated regulation of apoptosis-related proteins, FOXO3a was silenced by siRNA. As shown in Figure 6A, si-FOXO3a successfully reduced the protein level of FOXO3a in DU145 and PC-3 cells. After FOXO3a knockdown, the effect of SAHA on Bcl-2 family proteins and survivin was partially abrogated (Figure 6A). si-FOXO3a caused a partial rebound of Bcl-2 and survivin upon SAHA treatment, compared with the si-control group, whereas knockdown of FOXO3a only slightly increased Bim and Bax expression levels after SAHA treatment (Figure 6A). The quantitative data from Western blotting are shown in Figure 6B and 6C. The increased levels of Bcl-2/Bax ratio were observed after silencing of FOXO3a (Figure 6B, 6C). These data illustrate that FOXO3a plays an important role in SAHA-induced apoptosis in prostate cancer cells.

Discussion

The findings of this study have shown that treatment with the histone deacetylase (HDAC) inhibitor, suberoylanilide hydroxamic acid (SAHA), promoted apoptosis via the Akt/FOXO3a signaling pathway in prostate cancer cells *in vitro*. These findings are of interest because prostate cancer is the second most common cancer in men in the Western world [1]. Traditionally, patients with prostate cancer were treated with androgen-deprivation therapy (ADT), which could be achieved either through medical or surgical castration [5]. However, progression to castration-resistant prostate cancer (CRPC) commonly occurs. Taxane-based chemotherapy is the most common treatment of CRPC currently, but it is severely limited by drug resistance, dose-limiting toxicities and nonresponders [22,23]. The findings of this study are supported by previous studies that have shown that the HDAC inhibitor, SAHA, suppressed cell proliferation in prostate cancer cells *in vitro* and *in vivo* [15].

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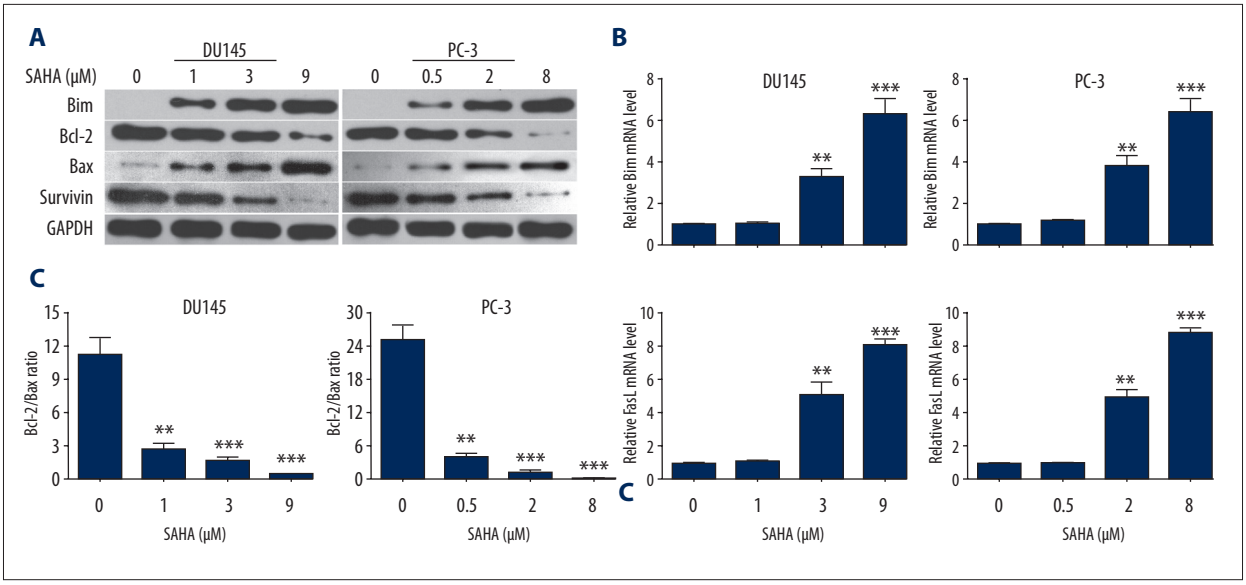

Figure 5. Western blotting and quantitative polymerase chain reaction (qPCR) analysis of apoptosis-related proteins in DU145 and PC-3 cells. (**A**) DU145 cells were treated with four doses of SAHA (0, 1, 3, 9 μM) for 48 h. PC-3 cells were treated with four doses of SAHA (0, 0.5, 2, 8 μM) for 48 h. Protein levels of Bim, Bcl-2, Bax and survivin were analyzed by Western blotting. GAPDH served as a loading control. Data are representative images of three independent experiments. (**B**) The mRNA levels of Bim and FasL were determined by qPCR. GAPDH served as an internal control for qPCR. (**C**) Quantitative data of Western blotting showed the reduction of Bcl-2/Bax ratio upon SAHA treatment. Data were presented as the mean ± standard deviation (SD) and performed in triplicate. ** $P<0.01$, *** $P<0.001$ versus the control group.

In this study, the MTT cell proliferation assay confirmed that SAHA decreased cell viability of DU145 and PC-3 cells in a dose-dependent manner (Figure 1). This finding is consistent with a previously reported study that demonstrated that SAHA induced apoptosis in different prostate cancer cell lines [24]. Also, cell cycle analysis in this study also showed that SAHA treatment resulted in the increase in cells in the sub-G_0/G_1 phases in a dose-dependent manner (Figure 2). This data further confirmed the results of the MTT cell proliferation assay, since it is accepted that the increase in the sub-G_0/G_1 population is indicative of apoptosis. Also, the Annexin-V-FITC/PI staining confirmed that SAHA caused cell apoptosis in both DU145 and PC-3 cells (Figure 3). Taken together, the findings from these experiments have shown that SAHA treatment caused cell apoptosis in DU145 and PC3 cells in a dose-dependent manner.

The findings suggest that G2/M arrest could prevent the cell with DNA damage from entering M phase. It is well established that cyclin-B/CDK1 plays a pivotal role in regulating G2 phase transition [25]. A recent study has shown that cyclin A2 is required for the activation of cyclin-B/CDK1 [26]. We have ishown that SAHA treatment reduced the protein levels of cyclin B and cyclin A2 in a dose-dependent manner (Figure 4), suggesting that downregulation of these two cyclins further leads to G2/M arrest in prostate cancer cells.

FOXO3a is a downstream substrate of Akt, and it mediates cell cycle arrest, DNA repair, and apoptosis [27]. Akt inactivates FOXO3a through post-translational modification. Briefly, activation of Akt increases phosphorylation of FOXO3a in the cytosol, and results in nucleus exclusion of FOXO3a. It has been reported that in prostate tumor specimens, phosphorylated FOXO3a is accumulated in the cytosol, in contrast to exclusively nuclear accumulation in normal prostate cells [28], indicating that FOXO3a is inactivated in prostate cancer. Also, FOXO3a plays a role in AR-independent progression of prostate cancer [29]. Therefore, the Akt/FOXO3a signaling pathway could be a promising therapeutic target for prostate cancer.

In this study, we demonstrated that SAHA treatment caused a significant reduction in p-Akt in prostate cancer cells, leading to a decrease of p-FOXO3a (Figure 4). These findings indicate that SAHA treatment blocked the inactivation of FOXO3a by activating Akt, with FOXO3a further exerting its pro-apoptotic effect in DU145 and PC-3 cells. A recent study reported that inhibition of Akt promotes FOXO3a-dependent apoptosis via inducing pro-apoptotic response-4 (Par-4) in prostate cancer [30]. Whether Par-4 is also involved in SAHA-induced apoptosis needs further investigation. However, it has been reported that FOXO3a increases the expression of AR in the prostate cancer cell line, LNCaP, suggesting that the Akt/FOXO3a signaling pathway works in concert with AR to regulate cell growth and cell death [31]. Further studies are required to determine

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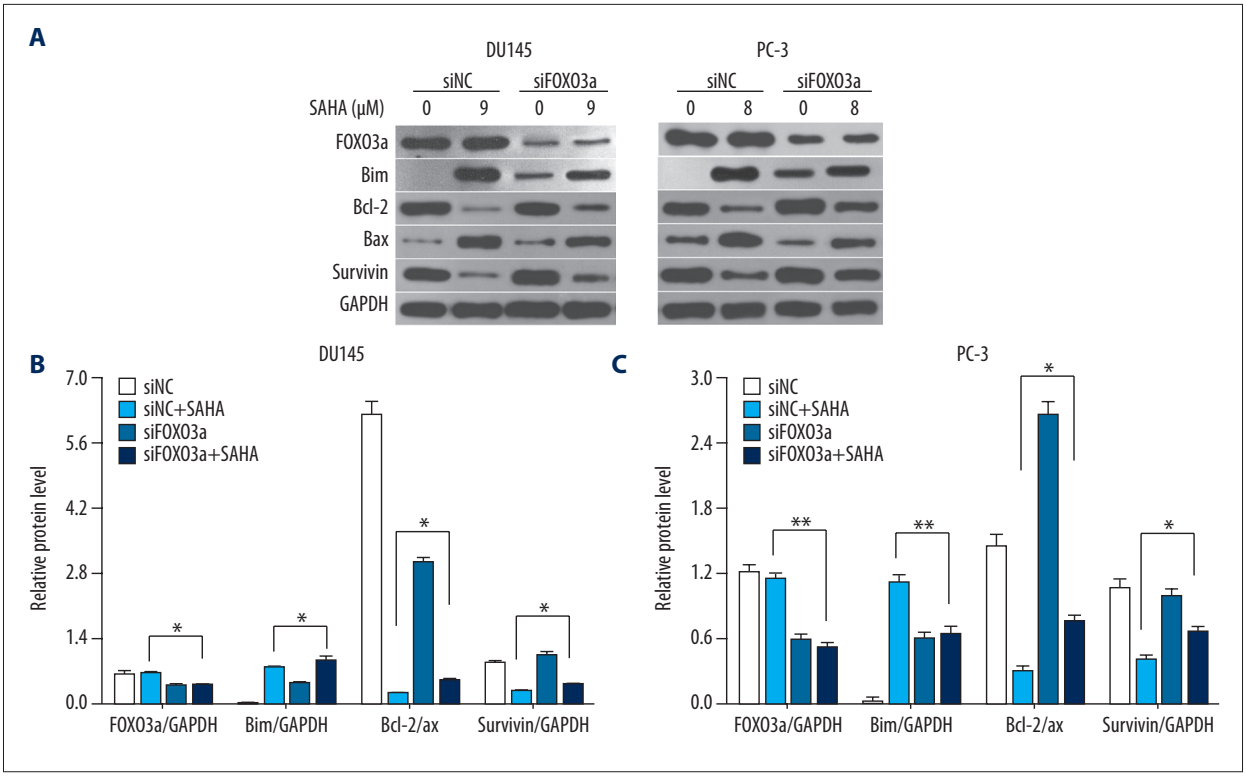

Figure 6. Role of FOXO3a in suberoylanilide hydroxamic acid (SAHA)-mediated apoptosis in DU145 and PC-3 cells. (**A**) DU145 cells were transfected with si-ctrl and si-FOXO3a for 48h. After transfection, cells were washed twice by RPMI1640, and treated with control or SAHA (9 μM) for 48 h. PC-3 cells were transfected with si-ctrl and si-FOXO3a for 48 h. After transfection, cells were washed twice by RPMI1640, and treated with control or SAHA (9 μM) for 48 h. Protein levels of FOXO3a, Bim, Bcl-2, Bax and survivin were analyzed by Western blotting. GAPDH served as a loading control. Data are representative images of three independent experiments. (**B, C**) Quantitative data of Western blotting in DU145 and PC-3 cells. Data were presented as the mean ± standard deviation (SD) and performed in triplicate. * $P<0.05$, ** $P<0.01$ versus the corresponding control.

whether SAHA also targets AR through regulating FOXO3a in DU145 and PC-3 cells.

The findings of the FOXO3a knockdown experiments in this study suggest that FOXO3a is involved in SAHA-induced apoptosis in prostate cancer cells. FOXO3a-mediated apoptosis occurs via two main pathways: the intrinsic mitochondrial pathway and the extrinsic apoptosis pathway [13]. Bcl-2 family proteins are well-characterized regulators of apoptosis. In this study, we have reported that FOXO3a participates in SAHA-induced upregulation of Bim and Bax (pro-apoptotic proteins), as well as the downregulation of Bcl-2 (anti-apoptotic protein) (Figure 5A and 5C). These findings are consistent with previous reports that showed that FOXOs induces Bim expression in hematopoietic cells [32,33]. Also, the chemotherapeutic drug paclitaxel mediates induction of FOXO3a and upregulates Bim expression, thus leading to apoptosis in breast cancer cells [34]. Our data indicate that this pathway is also involved in SAHA-induced apoptosis in prostate cancer cells. Furthermore, knockdown of FOXO3a partially reduced the SAHA-mediated downregulation of the anti-apoptotic protein survivin (Figure 6). This finding may be explained by a previously published report that demonstrated that FOXO3a directly binds to the survivin promoter to suppress its expression in tumor cells [22]. Apart from the intrinsic apoptosis pathway, the results of the quantitative polymerase chain reaction (qPCR) in this study also suggest that the extrinsic apoptosis pathway is involved in SAHA-induced apoptosis since the pro-apoptotic factor FasL was upregulated following SAHA treatment (Figure 5B).

Conclusions

The findings of this study showed that treatment with the HDAC inhibitor, SAHA, promoted apoptosis via the Akt/FOXO3a signaling pathway in prostate cancer cells *in vitro*.

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