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A New Histone Deacetylase Inhibitor, MHY4381, Induces Apoptosis via Generation of Reactive Oxygen Species in Human Prostate Cancer Cells

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Abstract

Histone deacetylase (HDAC) inhibitors represent a novel class of anticancer agents, which can be used to inhibit cell proliferation and induce apoptosis in several types of cancer cells. In this study, we investigated the anticancer activity of MHY4381, a newly synthesized HDAC inhibitor, against human prostate cancer cell lines and compared its efficacy with that of suberoylanilide hydroxamic acid (SAHA), a well-known HDAC inhibitor. We assessed cell viability, apoptosis, cell cycle regulation, and other biological effects in the prostate cancer cells. We also evaluated a possible mechanism of MHY4381 on the apoptotic cell death pathway. The IC_{50} value of MHY4381 was lower in DU145 cells (IC_{50}=0.31 μM) than in LNCaP (IC_{50}=0.85 μM) and PC-3 cells (IC_{50}=5.23 μM). In addition, the IC_{50} values of MHY4381 measured in this assay were significantly lower than those of SAHA against prostate cancer cell lines. MHY4381 increased the levels of acetylated histones H3 and H4 and reduced the expression of HDAC proteins in the prostate cancer cell lines. MHY4381 increased G2/M phase arrest in DU145 cells, and G1 arrest in LNCaP cells. It also activated reactive oxygen species (ROS) generation, which induced apoptosis in the DU145 and LNCaP cells by increasing the ratio of Bax/Bcl-2 and releasing cytochrome c into the cytoplasm. Our results indicated that MHY4381 preferentially results in antitumor effects in DU145 and LNCaP cells via mitochondria-mediated apoptosis and ROS-facilitated cell death pathway, and therefore can be used as a promising prostate cancer therapeutic.

Key Words: HDAC inhibitor, MHY4381, Prostate cancer, Apoptosis, Reactive oxygen species

INTRODUCTION

Prostate cancer is the most common type of cancer diagnosed in men. In 2018, prostate cancer represented 19% of all cancer diagnoses in men in the United States, which is the highest in the entire world (Siegel *et al*., 2018). The current front-line therapies for prostate cancer are either surgical removal of the tumor or radiation therapy, regardless of androgen sensitivity (Balk and Knudsen, 2008; Schröder *et al*., 2012). The androgen receptor (AR) is a transcription factor that plays a pivotal role in the regulation of androgen levels in tumors, and in the development of advanced prostate cancer (Balk, 2009). However, the mechanisms underlying the initiation and progression of prostate cancer are still not fully understood. In the early stages of prostate cancer, patients usually receive anti-androgen therapy; however, relapses occur frequently within 1 to 3 years, and patients then require treatment with a continuous conventional therapy. The effectiveness of chemotherapy remains limited, and is associated with serious adverse effects and a compromised quality of life (Keizman and Eisenberger, 2010; Kuban *et al*., 2013). Owing to increased mortality rates and failure of conventional chemotherapy in advanced prostate cancer patients, there is an urgent need for new alternative therapies (Bilusic *et al*., 2017;

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Litwin and Tan, 2017).

Several studies have indicated that epigenetic aberrations can contribute to cancer development (Ducasse and Brown, 2006; Dokmanovic *et al*., 2007; Park and Han, 2019). Histone protein acetylation is controlled by the balance of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Altered histone protein acetylation regulates the transcription of genes, particularly different types of oncogenes, tumor suppressor genes, and DNA repair genes (Dokmanovic and Marks, 2005; Yoon and Eom, 2016). Hence, abnormal expression of HDACs is coupled with the development of cancer (Mottet and Castronovo, 2008). Currently, the study of HDAC inhibitors is focused on their potential as emerging drugs in cancer therapy. However, the exact mechanisms by which individual HDACs regulate tumorigenesis are quite diverse. Four HDAC classes have been identified. Class I HDACs (HDAC1, 2, 3, and 8) are principally located in the nucleus and have uniform expression in all tissues, including prostate tissue (Weichert *et al*., 2008). Class II HDACs (HDAC4, 5, 6, 7, 9, and 10) are expressed in both the nucleus and cytoplasm of tissues. Class IV consists of HDAC11 only, which exhibits the properties of both class I and II HDACs (Perry *et al*., 2010). Overexpression of HDAC1, 2 and 3 have been reported in prostate cancer (Waltregny *et al*., 2004; Lin *et al*., 2013). Nevertheless, there are relatively few highly selective HDAC inhibitors, but a few drugs with proposed selectivity for several class I and class II HDACs have been developed. Pracinostat belongs to a class of hydroxamic acids that specifically target HDAC class I, II, and IV, and is currently in phase II of clinical trials for the treatment of prostate cancer patients (Ganai, 2016; Eckschlager *et al*., 2017). The relative abilities of pan- and selective-Class I HDAC inhibitors to attenuate androgen receptor (AR)-mediated target gene expression and proliferation were assessed in several prostate cancer cell lines (Ruscetti *et al*., 2016; McLeod *et al*., 2018). Additionally, HDAC inhibitors disrupt cytoplasmic AR via HDAC6 inhibition, leading to AR degradation and growth suppression of prostate cancer cells. Therefore, HDAC inhibitors can also be important therapeutic agents in castration-resistant prostate cancer (CRPC). However, current HDAC inhibitors are not effective in clinical trials treating CRPC.

The aim of this study was to evaluate the differential anticancer efficacy of the new HDAC inhibitor MHY4381 in both androgen-sensitive and androgen-independent cancer prostate cells. Our results suggest that the MHY4381 preferentially results in antitumor effects in DU145 and androgen-dependent LNCaP cells via both mitochondria-mediated apoptosis and reactive oxygen species (ROS)-facilitated cytotoxicity.

MATERIALS AND METHODS

Reagents

Suberoylanilide hydroxamic acid (SAHA) and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). MHY4381 (purity, 98.5%) was synthesized by Prof. Moon (College of Pharmacy, Pusan National University, Busan, Korea). Stock solutions (10 mM) of drugs were prepared in sterile dimethyl sulfoxide (DMSO) and stored at –20°C until use. Culture media and fetal bovine serum (FBS) were purchased from Gibco Invitrogen Corporation (Carlsbad, CA, USA). Primary antibodies against acetyl-H3, acetyl-H4, Bax, poly-ADP-ribose polymerase (PARP), Bcl-2, p53, cytochrome C, cyclin A, cyclin B, cyclin D, HDACs, caspase-3 and 9, and β-actin were purchased from Cell Signaling (Beverly, MA, USA). Antibodies against p27 and p21 and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) and DAPI (4'-6-diamidino-2-phenylindole) were purchased from Thermo Fisher Scientific, Inc (Invitrogen, MA, USA), while DCFH-DA (2',7'-dichlorofluorescein-diacetate) was obtained from Sigma-Aldrich.

Cell lines and culture conditions

Human prostate cancer cell lines (LNCaP, PC-3, and DU145) were obtained from the American Type Culture Collection (Manassas, VA, USA). DU145 and LNCaP cells were grown in RPMI 1640 (with 10% FBS), and PC-3 cells were maintained in F12 media supplemented with 4 mmol/L l-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cells were maintained as monolayers in a humidified atmosphere containing 5% CO_2 at 37°C, and the culture medium was replaced every two days.

Total HDAC enzyme activity assay

HDAC enzyme activity was assessed using an HDAC fluorogenic assay kit (BPS, Bioscience, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, HDAC enzymes were incubated with vehicle, various concentrations of MHY4381, or SAHA at 37°C for 30 min in the presence of an HDAC fluorometric substrate. Fluorescence was measured using VICTOR 3 (PerkinElmer, MA, USA) with excitation at 360 nm and emission at 460 nm. Data were analyzed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Cytotoxicity assay

The cytotoxic effects of MHY4381 and SAHA were assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, 5 mg/mL). Approximately, 3×10^3 cells per well were seeded into a 96 well plate. After 24 h, cells were treated with a range of drug concentrations (0.01-10 μM) for 24 and 48 h. One hundred microliters of MTT solution was added to each well at the end of the experiment and incubated for 4 h at 37°C in the dark. A VERSA Max Microplate Reader (Molecular Devices Corp., CA, USA) was then used to measure the optical density at 540 nm. The data obtained from three independent experiments were used to calculate the IC_{50} from sigmoidal dose-response curves using SigmaPlot 10 software (Jandel Scientific, San Rafael, CA, USA).

Colony formation assay

A total of 500 cells/well were seeded into 6 well plates and then incubated with MHY4381 (1 μM) or SAHA (1 μM) for 14 days. Viable colonies were fixed with methanol, stained with 0.05% crystal violet for 20 min, washed with phosphate buffered saline (PBS), and air dried. Colonies with more than 30 cells were counted and then normalized to the numbers in the control group (Franken *et al*., 2006).

Western blot analysis

After 48 h of treatment with MHY4381 (0.1, 0.5, or 1 μM) or SAHA (1 μM), cells were collected and washed with cold PBS. Total protein was isolated using a PRO-PREP™ pro-

tein extraction solution (Bio-Rad, Hercules, CA, USA). Equal amounts of protein (20 μg) were loaded onto 6-15% SDS polyacrylamide gels and then transferred to PVDF membranes. The membranes were then incubated with the desired primary antibodies at 4°C overnight, washed with TBS buffer, and then incubated with HRP-conjugated anti-mouse or anti-rabbit antibodies. The immunoreactive bands were visualized using Chemi Doc™ (Bio-Rad, Hercules, CA, USA), and the blots were quantitatively analyzed using an image analyzer (LAS-4000; Fuji film, Tokyo, Japan).

Cell cycle analysis by flow cytometry

Cells were seeded and allowed to attach overnight, and were then treated with MHY4381 (0.1, 0.5 or 1 μM) or SAHA (1 μM) for 48 h. The total number of cells, including the ones in suspension and those adhered to the walls of the wells, were harvested separately to identify sub-G1 or other cell cycle stages, respectively, and were washed in 1% bovine serum albumin (BSA) before fixing in 95% ice-cold ethanol containing 0.5% Tween-20 for 1 h. Cells (1×10^6) were washed in a solution containing 1% BSA, stained with cold propidium iodide (PI) staining solution (10 μg/mL PI and 100 μg/mL RNase in PBS), and incubated in the dark for 30 min at room temperature. Data were analyzed using a flow cytometer (Guava EasyCyte flow cytometer, Millipore, Billerica, MA, USA).

AnnexinV-FITC binding assay

The AnnexinV-FITC binding assay was carried out using the AnnexinV-FITC detection kit (BD, Bioscience). The cells were treated with MHY4381 (0.1, 0.5, or 1 μM) or SAHA (1 μM) for 24 and 48 h. The samples were prepared according to the manufacturer's instructions and analyzed by flow cytometry (Guava EasyCyte flow cytometer, Millipore).

4′-6-Diamidino-2-phenylindole (DAPI) staining

Morphological changes in the nuclear chromatin in apoptotic cells were assessed by DAPI staining. Cells (1×10^4) were seeded into a 6-well plate dish and treated with MHY4381 (0.1, 0.5, or 1 μM) or SAHA (1 μM) for 48 h, followed by fixation with methanol and staining with DAPI solution (1 μg/mL). The staining solution was discarded, and the cells were washed with cold PBS. Confocal microscopy was performed using an FV10i microscope (40x, Olympus, Tokyo, Japan) to visualize apoptotic cells.

Intracellular ROS assay

A non-fluorescent 2',7'-dichlorofluorescein-diacetate (DCFH-DA, 10 μM) intracellular probe was used to detect intracellular ROS formation. Approximately 1×10^3 cells/well were seeded into a 96-well plate. After 24 h, the cells were exposed to MHY4381 (1 μM) or SAHA (1 μM) for 12 h followed by incubation with 10 μM DCFH-DA in serum-free media for 30 min at

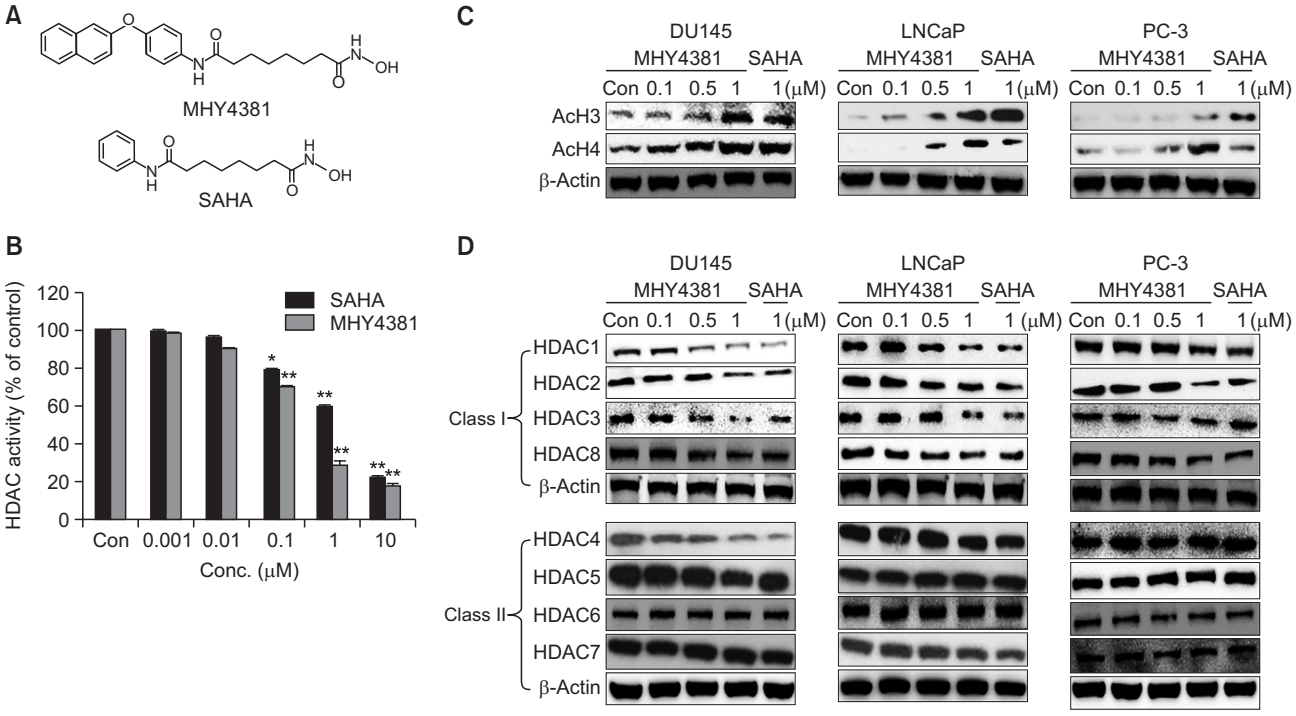

Fig. 1. Effect of MHY4381 and SAHA on histone deacetylase (HDAC) expression and activity and acetylation of histones (H3 and H4). (A) The chemical structures of MHY4381 and SAHA. (B) HDAC enzyme activity was measured using a fluorogenic HDAC assay kit. HDAC enzyme activity is shown relative to that of the control. Three independent experiments were performed and results are expressed as mean ± SD. Statistical analysis was performed using one-way analysis of variance, followed by Bonferroni's multiple comparison tests. *p<0.05 and **p<0.01 indicate significant differences between the control and treatment groups. (C) Effect of MHY4381 and SAHA on the levels of acetylated H3 and H4. Prostate cancer (DU145, LNCaP, and PC-3) cells were exposed to the indicated concentrations of MHY4381 and SAHA for 48 h. β-Actin was used as a loading control. (D) Effect of MHY4381 and SAHA on the protein expression levels of HDACs. Prostate cancer (DU145, LNCaP, and PC-3) cells were exposed to the indicated concentrations of MHY4381 and SAHA for 48 h and expression of HDACs (class I and II) was measured by western blot analysis. β-Actin was used as a loading control.

37°C. The cells were washed twice with PBS, and DCF fluorescence was detected using a fluorometric imaging plate reader at excitation and emission wavelengths of 488 nm and 520 nm, respectively. To observe the fluorescence image, the cells were seeded into a confocal dish and treated as described previously with the given compounds (MHY4381 or SAHA, each 1 μM) and DCFH-DA in serum-free media for 30 min at 37°C. After washing the cells with PBS, their images were captured using an FV10i microscope (Olympus).

Statistical analysis

All the data are presented as mean ± SD from at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni's test. Analysis was performed using Graph Pad Prism Software Version 5.0 (GraphPad Software). A *p*-value of <0.05 was considered statistically significant.

RESULTS

MHY4381 inhibits HDAC enzyme activity and expression in prostate cancer cells

The chemical structures of MHY4381 and SAHA are shown in Fig. 1A. Both MHY4381 and SAHA treatment significantly inhibited HDAC enzyme activity in a concentration-dependent manner, and the IC_{50} value (0.31 μM) for MHY4381 was considerably lower than that of SAHA (1.21 μM) (Fig. 1B). The effect of MHY4381 on the expression levels of acetylated histone proteins and HDACs was measured in three prostate cancer cell lines (DU145, LNCaP, and PC-3) by western blotting. MHY4381 treatment markedly increased acetylated H3 and H4 protein levels in both DU145 and LNCaP cells at 0.5 μM and 1 μM (Fig. 1C). In PC-3 cells, hyperacetylation of H3 and H4 was observed at 1 μM MHY4381 (Fig. 1C). After 48 h of MHY4381 treatment, class I HDACs (1-3 and 8) were noticeably downregulated in the three prostate cancer cell lines. In contrast, the expression of HDAC4 (class II) was downregulated only in DU145 cells but was unchanged in both LNCaP and PC-3 cells by MHY4381 treatment. The expression of the other class II HDACs (5, 6, and 7) was unaffected by treatment with MHY4381 in all three prostate cancer cell lines (Fig. 1D).

MHY4381 inhibits the proliferation of prostate cancer cells

The cytotoxicity of MHY4381 in the human prostate cancer cell lines (DU145, LNCaP, and PC-3) was assessed by MTT assay. Both MHY4381 and SAHA prevented cancer cell

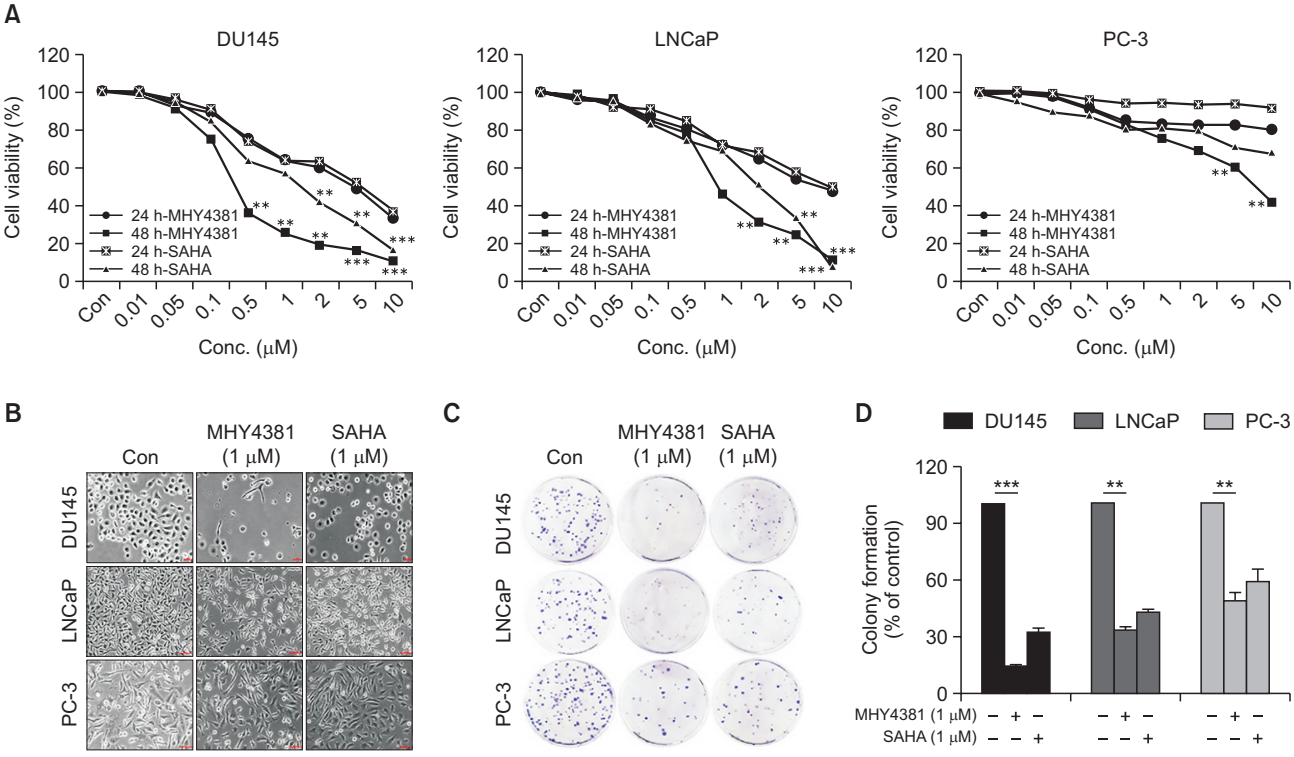

Fig. 2. Effect of MHY4381 and SAHA on viability and morphology of prostate cancer (DU145, LNCaP, or PC-3) cells. (A) The cells were treated with MHY4381 or SAHA at various concentrations (0.01-10 μM) for 24 h and 48 h. Cell viability was assessed using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, and the data represent the mean ± SD of three independent experiments. (B) Morphological changes (cell body shrinkage and reduction of cell number) in the cells were documented by photography after MHY4381 (1 μM) and SAHA (1 μM) treatment for 48 h. Light microscopic images representative of three independent experiments are shown. Scale bar, 100 μm. (C) The effect of MHY4381 or SAHA on prostate cancer single cell proliferation assessed by colony formation assay. Prostate cancer cells were treated with MHY4381 (1 μM) or SAHA (1 μM), and allowed to form colonies in fresh medium for 14 days. (D) Representative histogram showing the plating efficiency percentage in prostate cancer cells. Data are expressed as mean ± SD (n=3). ***p*<0.01, ****p*<0.001 versus control group.

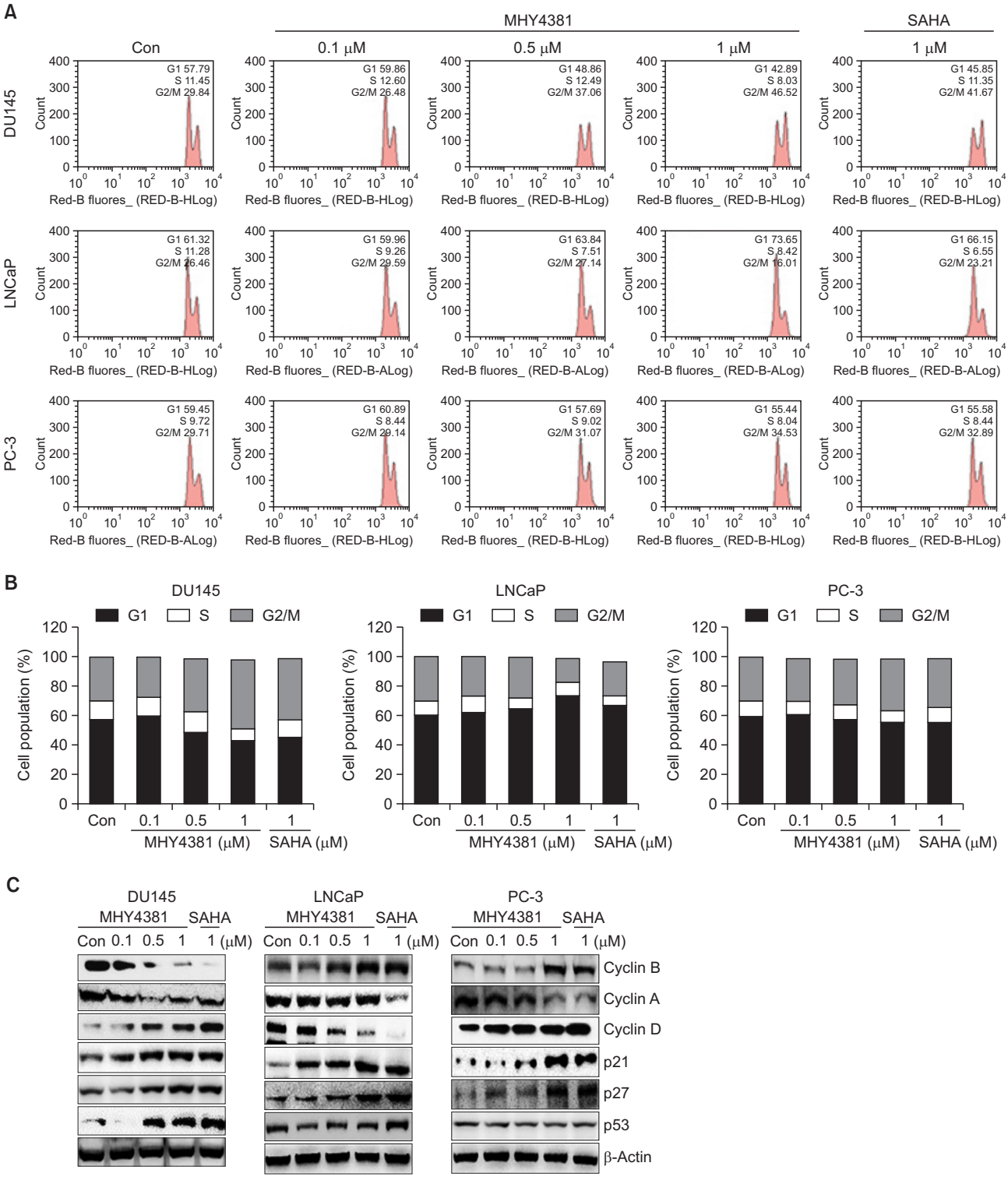

Fig. 3. Effect of MHY4381 or SAHA on cell cycle distribution in prostate cancer cells (DU145, LNCaP, and PC-3). (A) Cells were treated with MHY4381 or SAHA at the indicated concentrations for 48 h, stained with propidium iodide (PI) and analyzed by flow cytometry to determine the cell cycle distribution. (B) Bar diagram showing the G1, S, and G2/M phase distributions of prostate cancer cells treated with vehicle control, MHY4381, or SAHA. Data are expressed as mean (n=3). (C) Effect of MHY4381 or SAHA on the protein expression levels of cell cycle regulatory proteins. DU145, LNCaP, and PC-3 cells were treated with MHY4381 (0.1, 0.5, or 1.0 μM) or SAHA (1 μM) for 48 h. The expression levels of cyclin B, cyclin A, cyclin D, p21, p27, and p53 were detected by western blot analysis. Data are representative of three independent experiments. β-Actin was used as a loading control.

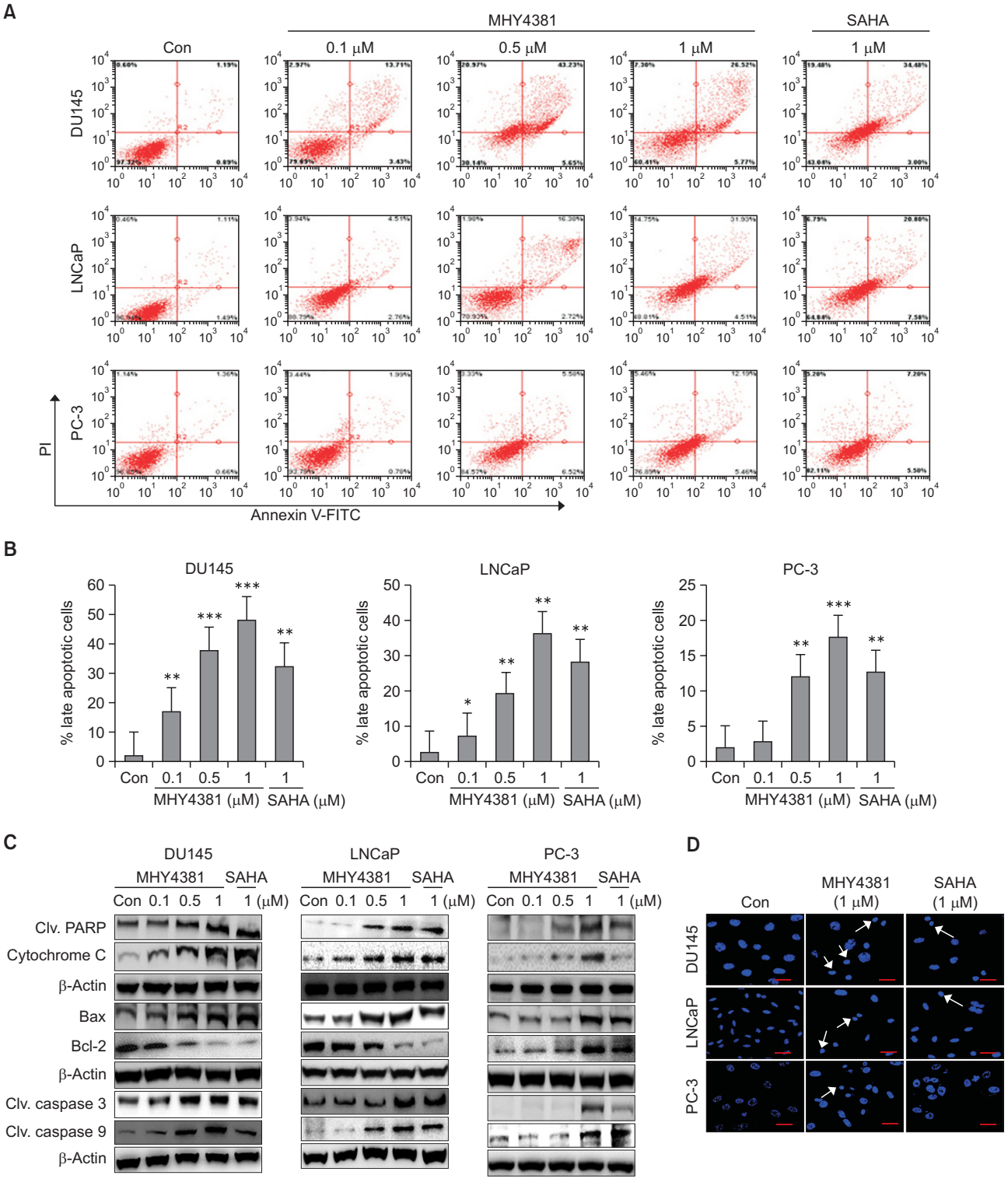

Fig. 4. Apoptosis induced by MHY4381 and SAHA in DU145, LNCaP, and PC-3 prostate cancer cells. (A) Cells were treated with vehicle control, MHY4381 (0.1, 0.5, or 1 μM), or SAHA (1 μM) for 48 h, then stained with FITC-conjugated Annexin V and propidium iodide (PI) for flow cytometry analysis. The flow cytometry profile represents AnnexinV-FITC staining on the x axis and PI staining on the y axis. (B) Bar graph showing the percentage of late apoptotic cells. Data are expressed as mean ± SD. *p<0.05, **p<0.01, and ***p<0.001 versus the control group of three independent experiments. (C) Cells were treated with vehicle control, MHY4381 (0.1, 0.5, or 1 μM), or SAHA (1 μM) for 48 h and the expression of apoptosis-related proteins was analyzed by western blot. β-Actin was used as a loading control. (D) Effects of MHY4381 or SAHA on nuclear morphological changes assessed by DAPI staining. DU145, LNCaP, and PC-3 cells were treated with MHY4381 (1 μM) or SAHA (1 μM) for 48 h, followed by fixation and DAPI staining. Images were observed using an Olympus confocal microscope (FV10i. Magnification ×400, Olympus). Scale bar, 50 μm.

proliferation in a concentration- and time-dependent manner. MHY4381 was more potent than SAHA (IC_{50}, 5.48 μM) with the following IC_{50} values in the different cell lines after treatment: DU145 (IC_{50}=0.31 μM), LNCaP (IC_{50}=0.85 μM), and PC-3 (IC_{50}=5.23 μM) (Fig. 2A). All three human prostate cancer cell lines showed different growth inhibition responses when exposed to MHY4381. Prominent morphological changes included cellular elongation induced by MHY4381 in both DU145 and LNCaP cells after 48 h of treatment (Fig. 2B).

MHY4381 inhibits colony formation in prostate cancer cells

To evaluate the anti-tumor effects of MHY4381, a colony formation assay was performed. The ability to form colonies is a fundamental characteristic of cancer cells (Nair *et al*., 2004; Franken *et al*., 2006). As shown in Fig. 2C and 2D, colony formation of DU145 cells (15% of control), LNCaP cells (34% of control), and PC-3 (49.17% of control) cells was significantly inhibited after treatment with MHY4381. The inhibitory potency of MHY4381 on colony formation was higher than that of SAHA.

Effect of MHY4381 on cell cycle regulation in prostate cancer cells

HDAC inhibitors reduce cellular growth by arresting the cell cycle at various checkpoints. The three prostate cancer cell lines were treated with MHY4381 (0.1, 0.5, or 1 μM) or SAHA (1 μM) for 48 h, and their DNA content was measured by flow cytometry. As shown in Fig. 3A, MHY4381 significantly induced G2/M accumulation in DU145 cells and simultaneously decreased the number of cells in S phase. In comparison, MHY4381 arrested cells at the G1 phase of the cell cycle in LNCaP cells. A similar effect was observed with SAHA treatment and occurred in a concentration-dependent manner. Both MHY4381 and SAHA arrested cells at the G2/M phase of the cell cycle in PC-3 cells, but this was not significant. The relative distribution of the effects on different phases of the cell cycle is shown in Fig. 3B. The effect of MHY4381 on the expression of cell cycle proteins was studied by western blotting. The expression levels of cyclin B and cyclin A were markedly decreased, whereas the expression of cyclin D was increased in DU145 cells after MHY4381 treatment. In LNCaP cells, the expression of cyclin D was reduced after MHY4381 treatment. MHY4381 considerably enhanced both $p21^{WAF1/CIP1}$ and p27 protein levels in DU145 and LNCaP cells, and to a lesser

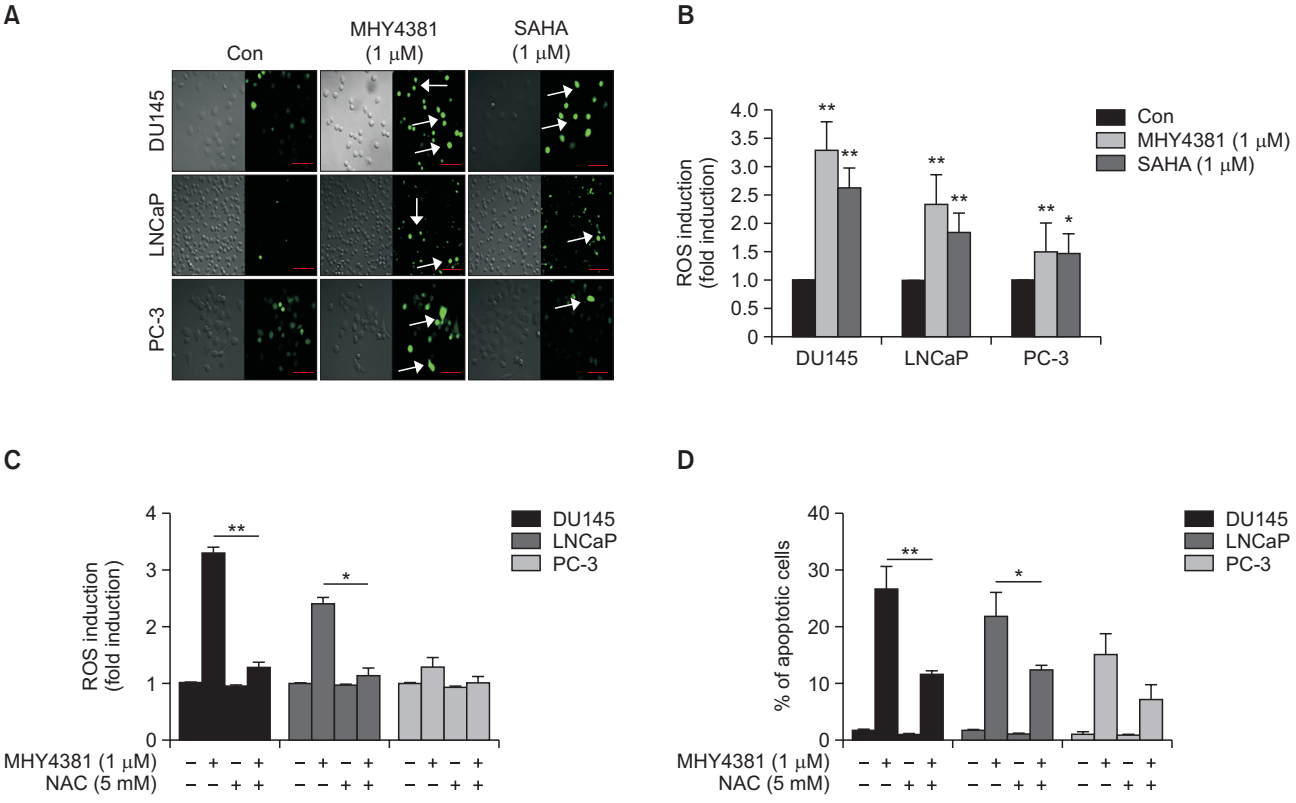

Fig. 5. MHY4381 and SAHA induce generation of ROS in prostate cancer cells. (A) DU145, LNCaP, and PC-3 cells were treated with the indicated concentrations of MHY4381 and SAHA for 12 h and then stained with DCFH-DA (10 μM) for 30 min. Images were obtained using an Olympus confocal microscope (FV10i. Magnification ×400, Olympus). Scale bar, 50 μm. (B) Cells were treated with the indicated concentrations of MHY4381 and SAHA for 12 h and, stained with DCFH-DA (10 μM) and absorbance was read using a fluorometric imaging plate reader. Bar graph showing the ROS levels. Data are expressed as mean ± SD. **p*<0.05, ***p*<0.01 versus control group for three independent experiments. (C) Bar graph showing the ROS level in cells after exposure to MHY4381 (1 μM), NAC (5 mM), or MHY4381 (1 μM) plus NAC (5 mM). **p*<0.05, ***p*<0.01 versus MHY4381. (D) Apoptosis induced by ROS generation observed by flow cytometry. After exposure to MHY4381 (1 μM), NAC (5 mM), or MHY4381 (1 μM) plus NAC (5 mM) for 12 h, the apoptotic cells were observed by flow cytometry and depicted in a bar graph. The data are expressed as mean ± SD. **p*<0.05, ***p*<0.01 versus MHY4381.

extent in PC-3 cells, in a concentration-dependent manner. Furthermore, p53 was also upregulated in DU145 and LNCaP cells, whereas the expression levels of p53 did not noticeably change in PC-3 cells after MHY4381 treatment (Fig. 3C).

MHY4381 induces apoptosis in prostate cancer cells

AnnexinV/PI double staining and western blot analysis were performed to understand the mechanism of prostate cancer cell growth inhibition after MHY4381 treatment. In both DU145 and LNCaP cells, the number of late stage apoptotic cells were increased in a concentration-dependent manner after MHY4381 treatment when compared to the control (Fig. 4A, 4B). The expression of apoptosis-related proteins was measured by western blotting. Treatment with MHY4381 increased the levels of cleaved PARP. MHY4381 also caused the cytoplasmic release of cytochrome c and decreased Bcl-2 expression levels in both DU145 and LNCaP cells in a concentration-dependent manner (Fig. 4C). Next, we used DAPI staining to analyze the apoptotic cells by confocal microscopy. The number of apoptotic cells with enhanced fluorescence increased after DAPI staining of MHY4381 treated cells (Fig. 4D). PC-3 cells did not show any significant increase in apoptotic cell death with FACS analysis.

MHY4381 increases intracellular ROS formation in prostate cancer cells

Evidence has shown that intracellular ROS play an important role in cell death by inducing apoptosis (Robert and Rassool, 2012). Accordingly, we measured intracellular ROS formation in prostate cancer cell lines after MHY4381 treatment. As shown in Fig. 5A, the intracellular ROS levels increased about 3.4-fold compared to the control in DU145 cells after MHY4381 treatment, whereas in LNCaP cells, ROS levels increased by about 2.4-fold. In PC-3 cells, there was no significant generation of intracellular ROS. These data support the hypothesis that MHY4381 treatment increases ROS production, and that it could be involved in inducing apoptosis in prostate cancer cell lines (Fig. 5B). To further confirm the role of ROS in inducing apoptosis, an ROS scavenger (NAC) was used. Pretreatment with NAC completely erased the production of ROS induced by MHY4381 treatment in the prostate cancer cell lines. In parallel, NAC also significantly attenuated MHY4381-mediated apoptosis. These data support the notion that the generation of ROS by MHY4381 treatment may play a role in inducing apoptosis (Fig. 5C, 5D).

MHY4381 decreases AR and PSA expression in prostate cancer cells

In both androgen-dependent and independent prostate cancer cells, AR plays an important role in growth and proliferation. The effect of MHY4381 on AR expression levels and its target gene PSA was examined in cell lysates. As shown in Fig. 6, high concentrations of MHY4381 caused a significant reduction in both AR and PSA levels in DU145 and LNCaP cells, although not in PC-3 cells. The data show that there is an inhibitory effect of MHY4381 on the AR signaling pathway in prostate cancer cells which may affect the overall biological function of the cells.

DISCUSSION

HDAC inhibitors induce cancer cell cycle arrest, apoptosis, and autophagy through the regulation of target gene expression by altering the acetylation status of chromatin and other non-histone proteins. Currently, there are a number of HDAC inhibitors in various stages of clinical trials, and some have been approved for use in cancer patients (Marks, 2010; Eckschlager *et al*., 2017). It has been indicated that HDACs may be essential for the maintenance of specific target genes required for survival and proliferation of cancer cells but not of normal cells (Ropero and Esteller, 2007). Taken together, expression of HDAC1, 2, and 3 is highly increased in the prostate carcinoma tissues compared with those of normal tissues (Weichert *et al*., 2008). Therefore, the development of highly potent and selective HDAC inhibitors is still necessary to delineate the associated biological effects for the complex signaling pathway of cancer therapy.

It has become clear that in antiandrogen-resistant cancers, the androgen receptor (AR) signaling axis is intact and is required for prostate cancer growth. Thus, there is a heightened interest in developing new drugs that function in part by down-regulating AR expression in prostate tumors. In the present study, we evaluated the anticancer effects of a novel HDAC inhibitor MHY4381 against three types of prostate cancer cell lines (DU145, LNCaP, and PC-3). Our data show that MHY4381 has potent anticancer activities at low doses, not only inhibiting HDAC activity, but also inducing apoptosis through cell cycle arrest and ROS generation in different types of prostate cancer cells. Histone protein acetylation was induced in DU145 and LNCaP cells by MHY4381 treatment at 0.5 and 1 μM, whereas in PC-3 cells, this effect was only observed at the highest concentration (1 μM). HDAC enzymes control the balance between the acetylation and deacetylation of histone and non-histone proteins, which normally regulate the expression of transcriptional factors. It has been reported that both class I and class II HDACs are present in prostate cancer cells at various levels (Waltregny *et al*., 2004). How-

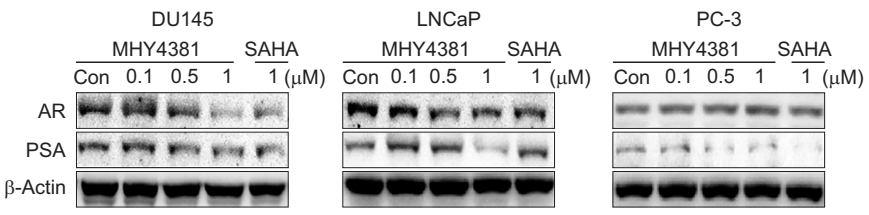

Fig. 6. Effects of MHY4381 and SAHA on the expression of the androgen receptor (AR) and prostate-specific antigen (PSA) in human prostate cancer cells. DU145, LNCaP, and PC-3 cells were treated with the indicated concentrations of MHY4381 and SAHA for 48 h. Western blotting using total protein lysate was used to assess the expression levels of AR and PSA. β-Actin was used as a loading control.

ever, the exact functional role of individual HDAC subfamilies on anticancer activity in prostate cancer remains unclear. In this study, we ascertained that MHY4381 significantly reduced the expression of HDAC1, HDAC2, and HDAC3 in DU145 and LNCaP cells, whereas HDAC2 and HDAC4 expression was only reduced marginally in PC-3 cells. Therefore, more specific inhibitors of HDAC1 and HDAC2 could be therapeutically useful for patients with prostate cancer. However, the mechanism behind HDAC1/HDAC2 inhibition and the reasons why DU145 and LNCaP cells are more susceptible to HDAC1 and 2 inhibitors than PC-3 cells are unknown. One of the main reasons for different susceptibilities of MHY4381 against prostate cancer cells is that it closely associated with cell cycle arrest at a specific checkpoint.

Previous research has shown that different types of HDAC inhibitors induce cell growth arrest via different intracellular signaling pathways; they discontinue the cell cycle at the G0/G1 or G2/M phase (Park *et al*., 2004; Shankar and Srivastava, 2008; Feng *et al*., 2015). Cell cycle progression can be regulated at both the G0/G1 and G2/M phases by the tumor suppressor p53 to recover DNA damage (Kastan and Bartek, 2004). Taken together, the cell cycle is known to be regulated by cyclins, CDKs, p16, p21, and p27 (Kastan and Bartek, 2004; Telles and Seto, 2012). While p53 contributes to many cell processes, its primary function is as a transcription factor; improper functioning of p53 can therefore cause loss of control of the cell cycle, leading to aberrant cell growth. It has been reported that checkpoint kinase 1 (Chk1) has a unique role in enhancing the resistance of normal cells against HDAC inhibitors both *in vitro* as well as *in vivo* (Lee *et al*., 2011). In this study, MHY4381 treatment caused an accumulation of cell in the G2/M phase in DU145 cells, whereas the G1 phase arrest was markedly increased in LNCaP cells. These results indicate that MHY4381 could inhibit prostate cancer cells growth via inducing cell cycle arrest at the G1 and G2/M phase. These results were very similar to previously reported studies that SAHA can cause G1 or G2/M arrest in different cancer cell lines (Butler *et al*., 2000; Komatsu *et al*., 2006). MHY4381 reduced the expression of cyclin A/B and increased the expression of p21 and p27 in DU145 cells. In contrast, cyclin D, which is involved in G1 phase arrest was reduced in LNCaP cells after MHY4381 treatment. Several studies have reported that $p21^{WAF1/CIP1}$ activation is mediated by HDAC inhibitors due to an enhancement of the acetylation of H3 and H4 histones associated with the $p21^{WAF1/CIP1}$ promotor (Sambucetti *et al*., 1999; Richon *et al*., 2000). Typically $21^{WAF1/CIP1}$ accumulates at the G1 transition, although it may also have a role in the G2/M transition (Niculescu *et al*., 1998). We observed that MHY4381 dramatically increased p21 expression in three prostate cancer cell lines, leading to cell cycle arrest. Upregulation of p21 can occur via both p53-dependent and p53-independent pathways (Zhao *et al*., 2006; Wang *et al*., 2012). In this study, MHY4381 upregulated p53 expression in the DU145 and LNCaP cells, but not PC-3 cells. Among the three cell lines we used, LNCaP or DU145 is more sensitive than PC3 to MHY4381-induced cell growth inhibition. We also found that antiapoptotic protein Bcl-2 levels play an important role in PC3 resistance because Bcl-2 proteins increased in PC-3 cells after MHY4381 treatment.

MHY4381 possesses the ability to stimulate ROS production and cytochrome c release to activate cell death, similar to what is seen for SAHA (Ruefli *et al*., 2001). A reduction in the levels of anti-oxidant enzymes such as MnSOD and catalase can lead to ROS accumulation, which in turn creates oxidative stress in cancer cells (Rosato *et al*., 2008). The activation of p53 and the accumulation of ROS are concurrent processes that are responsible for mitochondrial membrane disruption. MHY4381 significantly induced ROS production in DU145 and LNCaP cells, which enhanced apoptotic cell death. MHY4381-induced ROS production and apoptosis levels were dramatically protected by a combination with NAC in DU145 and LNCaP cells. Mitochondrial membranes are depolarized due to the translocation of Bax, resulting in the formation of apoptosomes with Apaf-1 and other procaspase proteins such as caspase-3 and 9 (Bishayee *et al*., 2015). HDAC inhibitors induce apoptosis mostly through the mitochondrial pathway by upregulating apoptotic proteins Bax, cleaved caspase-3, or cleaved caspase-9 expression (Shankar and Srivastava, 2008; Bao *et al*., 2016). In particular, MHY4381 significantly induced PARP cleavage and cytochrome c release. These data suggest that alteration ratio of Bax/Bcl-2 proteins lead to the release of cytochrome c, supporting that MHY4381 may induce the intrinsic apoptotic pathway through mitochondria.

It has been reported that AR is involved in regulating the cell cycle, and that inhibiting AR expression results in cell cycle arrest in cancer cells (Balk and Knudsen, 2008; Koryakina *et al*., 2015). Although the majority of human prostate cancer cell lines are AR-negative, detectable levels of AR mRNA have been reported in both DU145 and PC-3 cells (Xu *et al*., 2006). Androgen-regulated G1 transition has been observed in prostate cancer cells, where androgen-deprived prostate cancer cells generally arrest in the G2 phase through CDK4 activation (Shrotriya *et al*., 2012). Activated AR stimulates cyclin D1 and promotes Rb phosphorylation, which in turn stimulates G1 phase entry (Alimirah *et al*., 2006). Therefore, hyperactivity of AR and the loss/absence of p21 are important in establishing the resistance of prostate cancer cells towards apoptosis (Wang *et al*., 2001). We observed a reduction in AR expression levels in LNCaP and DU145 cells at higher concentrations of MHY4381. This result was similar to a previous study showed that a reduction in AR expression levels and an increase in p21 levels has been reported following SAHA treatment (Marrocco *et al*., 2007). Further study is required to understand the mechanism by which MHY4381 decreases AR expression in androgen-dependent prostate cancer cells.

In summary, our data demonstrated that the MHY4381 could inhibit growth of human prostate cancer cells through mitochondria-mediated apoptosis and a reduction in AR expression in DU145 and LNCaP cells. These cellular events are accompanied by modifying the activity of cell regulatory proteins via ROS production. Therefore, we suggest that MHY4381 could be used as an anticancer agent for the treatment of prostate cancer.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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