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A New Histone Deacetylase Inhibitor, MHY219, Inhibits the Migration of Human Prostate Cancer Cells via HDAC1

Umasankar De[1], Soma Kundu[2], Nabanita Patra[2], Mee Young Ahn[3], Ji Hae Ahn[1], Ji Yeon Son[1], Jung Hyun Yoon[2], Hyung Ryoung Moon[2], Byung Mu Lee[1] and Hyung Sik Kim[1,*]

[1]School of Pharmacy, Sungkyunkwan University, Suwon 440-746, [2]College of Pharmacy, Pusan National University, Busan 609-735, [3]Department of Oral & Maxillofacial Pathology, College of Dentistry, Wonkwang Bone Regeneration Institute, Daejeon Dental Hospital, Wonkwang University, Daejeon 302-120, Republic of Korea

Abstract

Histone deacetylase (HDAC) inhibitors are considered novel agents for cancer chemotherapy. We previously investigated MHY219, a new HDAC inhibitor, and its potent anticancer activity in human prostate cancer cells. In the present study, we evaluated MHY219 molecular mechanisms involved in the regulation of prostate cancer cell migration. Similar to suberanilohydroxamic acid (SAHA), MHY219 inhibited HDAC1 enzyme activity in a dose-dependent manner. MHY219 cytotoxicity was higher in LNCaP (IC_{50}=0.67 μM) than in DU145 cells (IC_{50}=1.10 μM) and PC3 cells (IC_{50}=5.60 μM) after 48 h of treatment. MHY219 significantly inhibited the HDAC1 protein levels in LNCaP and DU145 cells at high concentrations. However, inhibitory effects of MHY219 on HDAC proteins levels varied based on the cell type. MHY219 significantly inhibited LNCaP and DU145 cells migration by down-regulation of matrix metalloprotease-1 (MMP-1) and MMP-2 and induction of tissue inhibitor of metalloproteinases-1 (TIMP-1). These results suggest that MHY219 may potentially be used as an anticancer agent to block cancer cell migration through the repression of MMP-1 and MMP-2, which is related to the reduction of HDAC1.

Key Words: HDAC inhibitor, MHY219, MMPs, Migration, Prostate cancer

INTRODUCTION

Prostate cancer is estimated to be the highest cause of cancer-related deaths among males in Korea. It is also estimated that over 12,180 men are newly diagnosed with prostate cancer and over 1,620 patients died in 2013 in Korea (Jung *et al*., 2013). Prostate cancer development depends on male sex steroid hormone for growth and survival. Disruption of the male endocrine system during the developmental period may induce prostate cancer in experimental animals (Prins, 2008). In addition, insulin-like growth factor (IGF) and vitamin D also contribute to the development of prostate cancer (LeRoith *et al*., 1995). Despite considerable efforts to improve early detection and advances in chemotherapy, the high mortality rate of prostate cancer has markedly increased worldwide. Furthermore, prostate cancer metastasis remains a major challenge in clinical trials (Catalona, 1994). Thus, new chemotherapeutic agents are needed for the inhibition of prostate cancer metastasis.

Recently, histone deacetylase (HDAC) inhibitors emerged as a promising new class of anticancer agents that act through a variety of mechanisms, including growth inhibition, cell cycle arrest, differentiation, and apoptosis in cancer cell lines (Glozak and Seto, 2007; Emanuele *et al*., 2008; Fulda, 2008). The precise mechanism of HDAC inhibitors may also involve histone acetylation and deacetylation of lysine residues present in the tail of the core histones (Robbins *et al*., 2005). It was reported that HDAC activity was highly increased in metastatic cells compared with non-invasive cancer cells (Patra *et al*., 2013). In particular, HDAC1 was upregulated in hormone refractory prostate cancer and overexpression of HDAC1 led to an increase in prostate cancer cell proliferation (Halkidou *et al*., 2004a). Furthermore, HDAC4 may have an important role in the progression of androgen-independent hormone refectory prostate cancer because its nuclear accumulation coincides with the loss of androgen sensitivity (Seligson *et al*., 2005). Previously, our studies demonstrated that HDAC inhibitor, apicidin, inhibited migration and invasion of human endometrial

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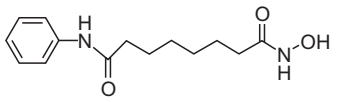
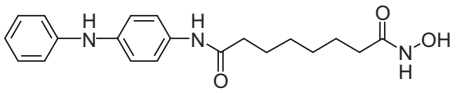
***Corresponding Author**
E-mail: hkims@skku.edu
Tel: +82-31-290-7789, Fax: +82-31-290-7798



Suberoylanilide hydroxamic acid (SAHA)



N1-hydroxy-N8-(4-(phenylamino)phenyl)octanediamide (MHY219)

Fig. 1. Chemical structure of 5-hydroxy-7-(oxiran-2-ylmethoxy)-2-phenyl-4H-chromen-4-one (MHY219) and SAHA.

cancer cells via HDAC4 (Ahn *et al*., 2012).

Proteolytic activation of matrix metalloproteinases (MMPs) in human cancer tissues is one of the critical steps against tumor invasion and metastasis (Kim *et al*., 2014). MMPs are a group of zinc-dependent endopeptidases, which play a pivotal role in the physiological regulation of the extracellular environment (Levy *et al*., 1998). SAHA was previously shown to inhibit breast cancer cell migration by inhibiting MMP-9 activity (Chiu *et al*., 2013). Moreover, a new HDAC inhibitor, MC133(S)-2 showed a potent effect against highly metastatic prostate cancer PC3 cells by decreasing MMP-9 activity and enhancing tissue inhibitor of metalloproteinases-1 (*TIMP-1*) mRNA levels (Laurenzana *et al*., 2013).

In this study, we synthesized MHY219 as a novel HDAC inhibitor, a hydroxamic acid derivative, by incorporating one phenylamino group in position 4 of the phenyl ring of SAHA (Fig. 1). The effects of MHY219 on prostate cancer cell migration were investigated and compared with that of SAHA.

MATERIALS AND METHODS

Cell lines and culture

Three human prostate cancer cell lines, LNCaP, PC3, and DU145, were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were grown in Roswell Park Memorial Institute medium (RPMI) 1640 (Gibco BRL, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL), 1.25 mM HEPES (Gibco BRL) and 1 mL of 100 U penicillin/streptomycin (Gibco BRL). Cells were maintained as monolayers in a humidified atmosphere containing 5% CO_2 at 37°C. The culture medium was replaced every 2 days. After 48 h of incubation, the culture medium was replaced with the treatment medium containing the desired concentration of the drugs.

HDAC1 activity assay

HDAC1 enzyme activity was assessed using a fluorogenic HDAC1 assay kit purchased from BPS Bioscience (San Diego, CA, USA) according to the manufacturer's instructions. Briefly, HDAC1 enzyme was incubated with vehicle or various concentrations of TsA, SAHA, or MHY219 at 37°C for 30 min in the presence of an HDAC1 fluorometric substrate. The HDAC1 assay developer (which produces a fluorophore in the reaction mixture) was added and the fluorescence was measured by using VICTOR 3 (PerkinElmer, Waltham, MA, USA) with excitation at 360 nm and emission at 460 nm. The measured activities were calculated by using GraphPad Prism (GraphPad software, San Diego, CA, USA).

Cell proliferation assay

A total of 5,000 cells were plated onto flat-bottomed 96-well plates and maintained overnight. Cells were incubated with 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 2 h. A volume of 200 μL of dimethyl sulfoxide (DMSO) was added and the absorbance per well was measured at 540 nm using the VERSA Max Microplate Reader (Molecular Devices Corp., Sunnyvale, CA, USA). Data were analyzed from three independent experiments then normalized to the absorbance of wells containing media only (0%) and untreated cells (100%). IC_{50} values were calculated from *sigmoidal* dose-response curves using *SigmaPlot* 10.0 (Systat Software Inc., San Jose, USA).

Migration assay

To perform the migration assay, 24-well modified Boyden chambers (Corning Life Sciences, Corning, New York, NY, USA) were used as described previously (Kim *et al*., 2000). Cells were seeded in the upper chambers at a density of 1 ×10^4 cells per well with RPMI 1640 containing 1% FBS and also media containing the desired concentrations of chemicals, FBS (5%) was added to the lower chambers. After 24 h incubation, the insert was washed with PBS and the cells on the upper side of the insert were removed by using a cotton swab. Cells on the lower side of the insert were fixed and stained with Diff-Quick solution (Baxter Healthcare Corp, Miami, FL, USA). The number of migrated cell was counted under a microscope (200× magnification) and the results were expressed as the percentage of invaded cells per field for each condition.

Western blot analysis

Cell pellets were resuspended in modified radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris (pH 7.8), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 15 mM $MgCl_2$, 1% NP-40, 0.5% sodium deoxycholate, 1 mM DTT, protease inhibitors 1:100, 20 mM *N*-ethylmaleimide) and further disrupted by mechanical shearing through a 19-gauge needle. Soluble proteins were then separated by centrifugation at 4°C for 5 min. Protein concentration was calculated using a Bio-Rad protein assay kit (Bio-Rad, Hemel Hempstead, UK). Proteins were run on a 10% SDS-PAGE gel, blotted onto nitrocellulose membranes, and incubated with the appropriate primary antibodies. The membranes were then incubated with horseradish-peroxidase labeled secondary antibody. The membranes were washed and developed with ECL Plus western blotting detection system (Amersham, Little Chalfont, UK).

Reverse transcription- polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIzol reagent (Gibco BRL) according to the manufacturer's protocol. A total of 2 μg RNA was reverse transcribed for 50 min at 42°C in a 20 μL reaction mixture containing 1 μL of the oligo primer (0.5 μg), 10 mM dNTP mixture, 25 mM $MgCl_2$ (4 μL), 0.1 M DTT (2 μL), RNase Out inhibitor (1 μL), superscript II (50 units) and 10× RT buffer (2 μL), followed by denaturation at 70°C for 15 min. The synthesized cDNAs were further amplified by PCR using specific primers. The primers sets were as follows: *MMP-1* (sense 5'-CGACTCTAGAAACACAAGAGCA-3', anti-sense

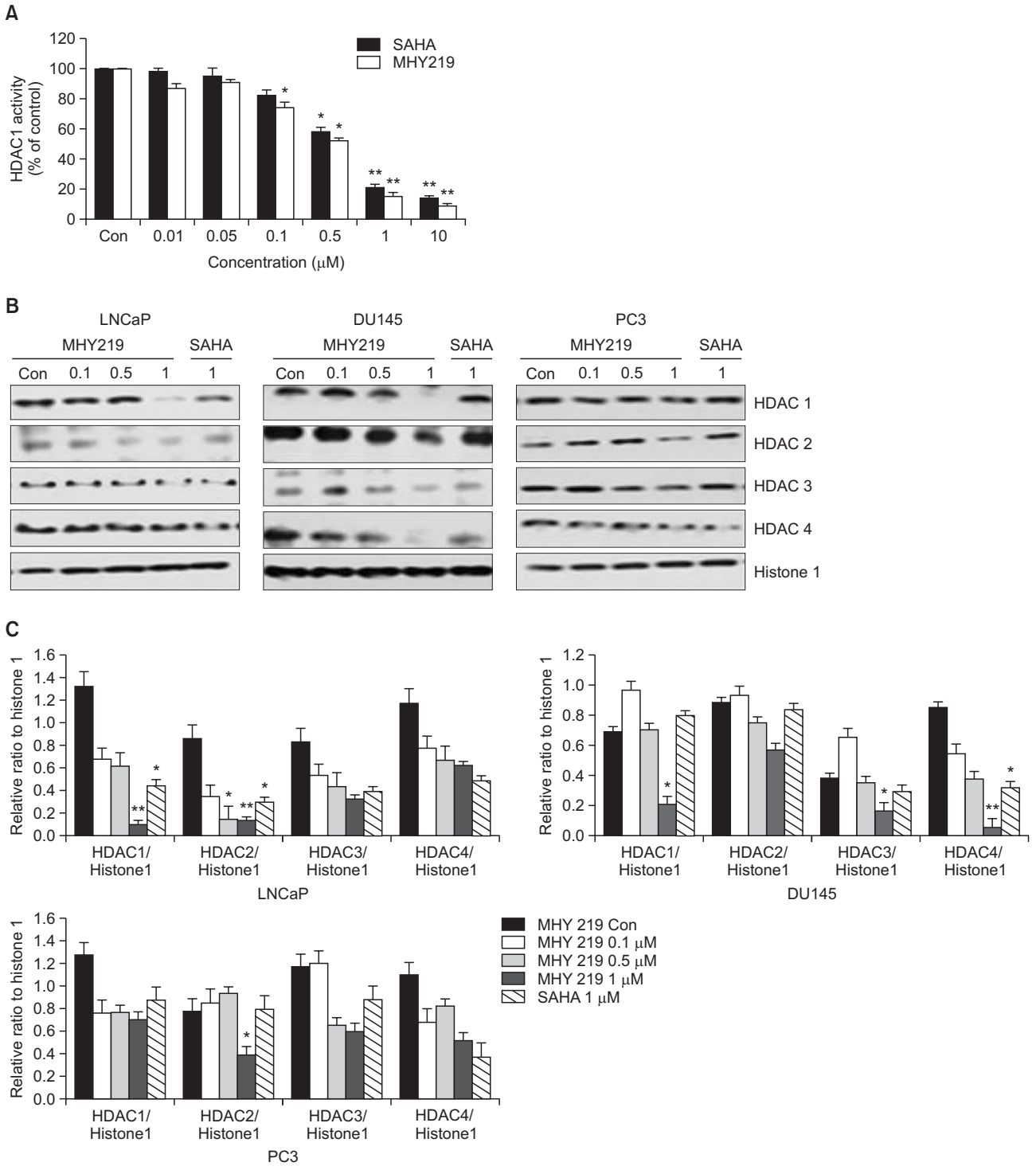


Fig. 2. Effect of MHY219 and SAHA on histone deacetylase (HDAC) 1 activity and expression levels of HDACs in prostate cancer cells. (A) Histone deacetylase 1 (HDAC1) enzyme activity was measured by using a fluorometric HDAC activity assay kit. This result represents the percentage of activity compared to control cells in each group, respectively. Results are expressed as mean ± standard error of the mean (S.E.M.) of three independent experiments. *p<0.05, **p<0.01 as determined by Student t-test compared to the control. (B) LNCaP, DU145, and PC3 cells were treated with the indicated concentrations of MHY219 and SAHA for 48 h and HDAC (class I and II) expression levels were measured by western blot analysis. Histone 1 was used as a housekeeping control protein. (C) Densitometric analysis of HDAC1, HDAC2, HDAC3, or HDAC4 ratio, respectively. Bars represent mean ± S.E.M of three independent experiments. *p<0.05, **p<0.01 as determined by Student t-test compared to the control.

5'-AAGGTTAGCTTACTGTCACACGCTT-3'); *MMP-2* (sense 5'-GTGCTGAAGGACACACTAAAGAAGA-3', anti-sense 5'-TTGCCATCCTTCTCAAAGTTGTAGG-3'); *MMP-9* (sense 5'-CACTGTTCCACCCCTCAGAGC-3', anti-sense 5'-GCCACTTGTCGGCGATAAGG-3'); *TIMP-1* (sense 5'-TGCACCTGTGTCCCACCCCACCCACAGACG-3', anti-sense 5'-GGCTATCTGGGACCGCAGGGACCCAGGT-3'); *GAPDH* (sense 5'-GGCGTCTTCACCACCATGGAG-3', anti-sense 5'-GCCTGCTTCACCACCTTCTT-3'). The cDNA was amplified in a 25 μL reaction mixture containing 10× PCR buffer (2.5 μL), 50 mM $MgCl_2$ (0.75 μL), 10 mM dNTP mixture (0.5 μL), and 20 μM of sense and anti-sense primers. The reaction was initiated at 94°C for 5 min and PCR was then performed using a variable number of the following amplification cycles: denaturation at 94°C for 45 sec, annealing at 56-66°C for 45 sec and extension at 72°C for 45 sec. The number of PCR cycles was estimated in a preliminary study and optimized in the PCR exponential phase. A final cycle of extension at 72°C for 5 min was also included. A 20 μL aliquot of each PCR product was analyzed by gel electrophoresis on a 2% agarose (w/v) gel. The molecular size of the amplified products was determined by comparison with molecular weight markers (100 bp DNA ladder, Intron, Seongnam, Korea) that were run in parallel with the RT-PCR products.

Statistical analysis

The data represent the mean ± SD from triplicate experiments performed in a parallel manner unless otherwise indicated. Statistical significance was assessed by using a paired Student's t-test. A $*p<0.05$ or $**p<0.01$ was considered statistically significant.

RESULTS

MHY219 inhibits HDAC1 enzyme activity

The effect of MHY219 on HDAC1 enzyme activity was examined using the fluorometric HDAC1 enzyme assay kit. As shown in Fig. 2A, MHY219 significantly inhibited HDAC1 enzyme activity in a concentration-dependent manner with an IC_{50} value of 0.276 μM, which was similar to that of SAHA (IC_{50}=0.254 μM). In our previous study, the effect of MHY219 on total HDAC enzyme activity was measured. As a result, MHY219 significantly inhibited total HDAC enzyme activity in a concentration-dependent manner (Patra *et al*., 2013). We next examined MHY219 inhibitory effect on the expression of specific HDACs isoforms in human prostate cancer cells. MHY219 effect on the expression of HDAC1, 2, 3, and 4 was identified by western blot analysis. A significant decrease in HDAC1 and HDAC2 levels was observed in LNCaP cells after MHY219 treatment. HDAC1, 2, 3, and 4 in DU145 cells were downregulated after MHY219 treatment. No change in HDAC1 was observed in PC3 cells (Fig. 2B).

MHY219 inhibits the proliferation of prostate cancer cells

MHY219 and SAHA significantly reduced the growth of human prostate cancer cells in a concentration- or time-dependent manner, as assessed by MTT assay (Fig. 3). MHY219 showed a more potent cytotoxic activity than SAHA against LNCaP, DU145, and PC3 cells with IC_{50} values of 3.98, 2.6, and >5 μM at 24 h and 0.97, 0.36, and 5.12 μM at 48 h, respectively. MHY219 and SAHA exhibited different sensitivity against the three prostate cancer cells lines. MHY219 and SAHA cytotoxicity was high in DU145 cells as compared to that in LNCaP and PC-3 cells.

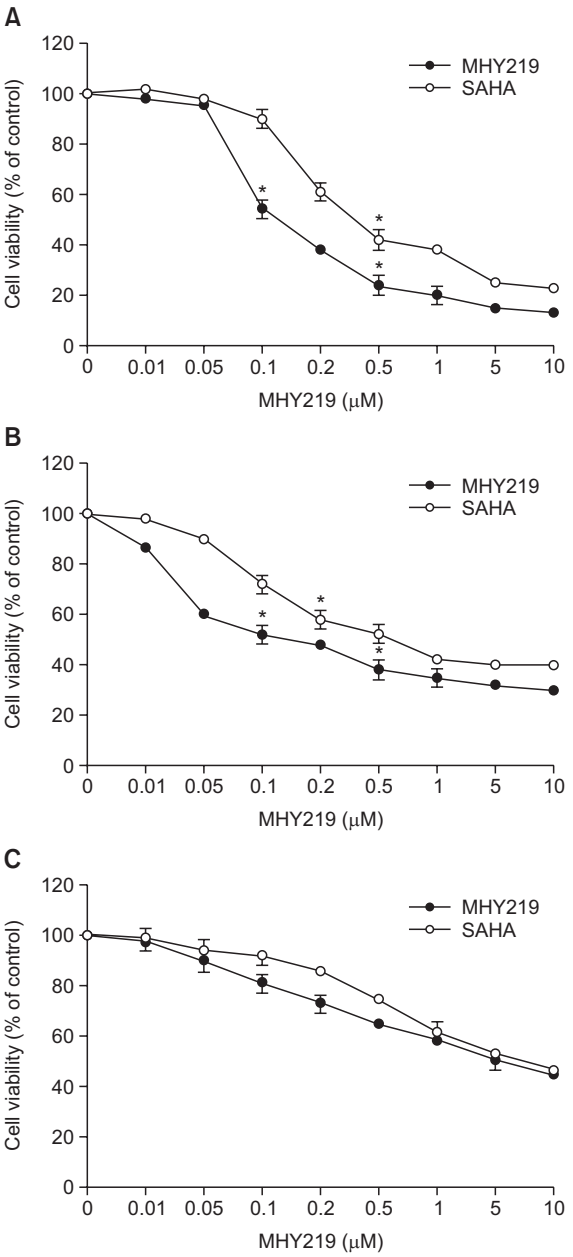


Fig. 3. Effect of MHY219 and SAHA on cell viability measured by MTT assay. (A) DU145 cells, (B) LNCaP cells, and (C) PC3 cells were treated with MHY219 and SAHA at various concentrations (0.01-10 μM/mL for 48 h. The percentage of viable cells was determined as the ratio between treated cells and untreated controls. Results were expressed as mean ± standard deviation (S.D.) of three independent experiments. $*p<0.05$ as determined by a Student t-test compared to the untreated control.

MHY219 inhibits the migration of prostate cancer cells

MHY219 inhibitory effect on the migration of prostate cancer cells (DU145, PC3, or LNCaP) was measured using a

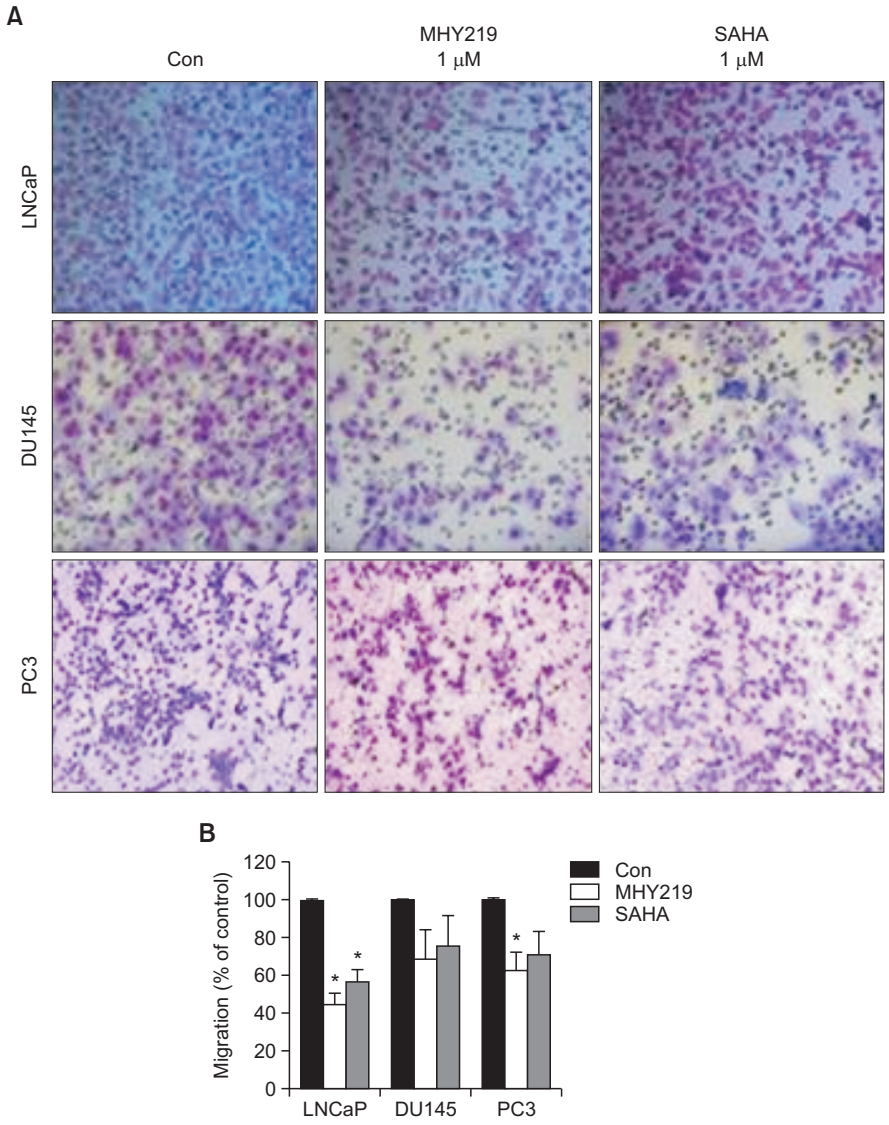


Fig. 4. Effect of MHY219 and SAHA on the migration of prostate cancer cells. (A) The cells were placed in the upper chamber inserts with the indicated concentrations of MHY219 and SAHA and allowed to migrate for 24 h. Membranes containing migrated cells were stained, and ten random fields from each experiment were counted under the microscope (×200). (B) Each bar represents the mean ± S.D. of three independent experiments. **p*<0.05 as determined by a Student *t*-test compared to the untreated control.

matrigel migration assay kit. Treatment with MHY219 (1.0 μM) and SAHA (1.0 μM) significantly reduced both LNCaP and PC3 cell migration, but not that of DU145 cells. The migration rate was significantly decreased up to 50% as compared with control in LNCaP cells, below the rate observed for SAHA (Fig. 4). Furthermore, MHY219 effect on metastasis promoting proteins such as MMP1, 2, and 9 was examined using western blot analysis. MHY219 and SAHA treatment significantly decreased MMP-1 and MMP-2 protein expression, but did not affect MMP-9 protein expression (Fig. 5A). MHY219 and SAHA significantly increased TIMP-1 expression levels in both LNCaP and PC3 cells. These data show that MHY219 decreases cellular migration through regulating MMP-1 and MMP-2 protein expression. To confirm the expression levels of migration-related protein expression after MHY219 treatment, we measured MMPs and *TIMP-1* mRNA levels in prostate cancer cells. As shown in Fig. 5B, *MMP-1* and *MMP-2* mRNA levels were significantly decreased after MHY219 treatment (0.5 and 1.0 μM). In addition, *TIMP-1* mRNA levels were markedly increased in a concentration-dependent manner after MHY219 treatment.

DISCUSSION

Cancer metastasis is the most important cause of cancer death and new anti-cancer agents that inhibit this process have been developed in preclinical studies. Uncontrolled degradation of the extracellular matrix and basement membrane, which is an essential part of the metastatic process, is believed to be associated with tumor cell migration and invasion (Liotta, 1986; Liotta *et al*., 1991). Recently, HDACs have

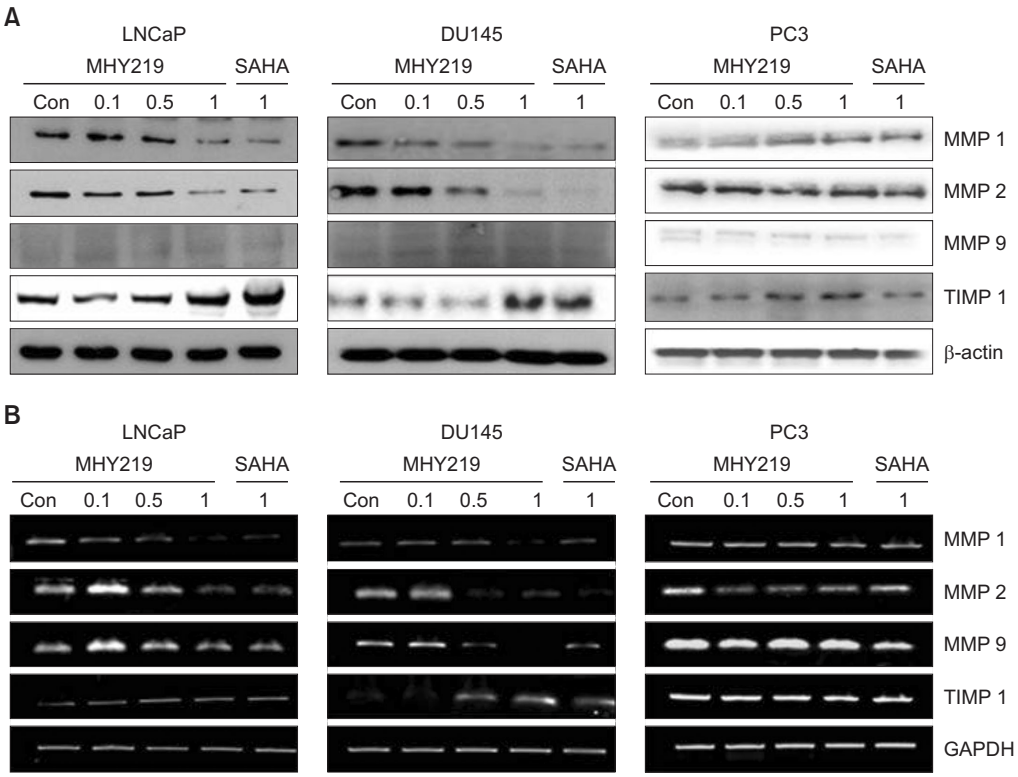


Fig. 5. Effects of MHY219 or SAHA on the expression of cancer cell migration-related genes in prostate cancer cells. (A) LNCaP, DU145, and PC3 cells were treated with the indicated concentrations of MHY219 and SAHA for 48 h and total protein was used to detect MMPs and TIMP-1 protein expression by western blot analysis. The protein levels were normalized to the levels of β-actin. (B) LNCaP, DU145, and PC3 cells were treated with the indicated concentrations of MHY219 and SAHA for 48 h and *MMP-1*, *MMP-2*, *MMP-9*, and *TIMP-1* mRNA levels were measured in prostate cancer cells treated with MHY219 or SAHA. Total RNA was isolated and RT-PCR was performed using the specific primers described in Materials and Methods. *GAPDH* was used as the housekeeping control gene. (C) Densitometric analysis of MMP1, MMP2, MMP9, and TIMP-1 ratio levels, respectively, on Western blots and (D) RT-PCRs. Results are expressed as mean ± S.E.M. of three independent experiments. **p*<0.05, ***p*<0.01 as determined by Student t-test compared to the control.

been shown to be important factors in migration or invasion of malignant cancer cells (Min *et al*., 2012; Seo *et al*., 2014; Zhang *et al*., 2014). Various HDAC inhibitors upregulate a set of metastasis suppressor genes or downregulate metastasis promoting genes, thereby, repressing cancer cell invasion and metastasis both *in vitro* and *in vivo* (Zhao *et al*., 2011; Chiu *et al*., 2013). In human prostate cancer cells, HDAC overexpression may be involved in the repression of growth suppressive genes, which is an important mechanism to promote cancer cell proliferation, migration, and invasion (Abbas and Gupta, 2008). Differential expression of HDAC1, HDAC2, and HDAC3 in prostate cancer plays a role during cancer progression (Weichert *et al*., 2008). In hormone refractory prostate cancer, HDAC4 is predominantly localized in the nucleus and plays an important role in cancer progression (Halkidou *et al*., 2004b).

This study investigated the effect of a novel HDAC inhibitor MHY219, a SAHA analog, on the inhibition of cell migration using three prostate cancer cell lines. MHY219 was previously shown to exert a broad spectrum of effects towards prostate cancer cells, including G2/M cell cycle arrest, p21 up-regulation, and induction of apoptosis as shown by the inhibition of Bcl-2, induction of PARP cleavage, and cytochrome c release (Patra *et al*., 2013). In this study, the sensitivity of prostate cancer cells to MHY219 varied from very sensitive LNCaP cells to low sensitive PC3 cells. The low sensitivity of PC3 cells to MHY219 is due to Bcl-2 overexpression, followed by resistance to HDAC inhibitor. MHY219 markedly reduced HDAC1 and HDAC3 expression in LNCaP and DU145 cells. However, HDAC1 and HDAC3 expression was not affected by MHY219 treatment in PC3 cells. We suggest that HDAC1 and HDAC3 may be responsible for the low cytotoxicity of MHY219. Therefore, proteasomal degradation of HDAC1 might explain the downregulation of HDAC1 in prostate cancer cells and further study will be needed. The target specificity of HDAC inhibitors remains unclear. However, it may be related to the different expression levels of specific HDACs observed in cancer cells. It is also possible that HDAC inhibitors have different effects on different cell types. Proteolysis of the extracellular matrix must occur in order for the cells to invade and migrate through the basement membrane. By regulating the gene expression of extracellular matrix related proteins; HDAC modulates the extracellular matrix composition, deposition, and removal and increases cancer cell invasion and migration.

MMP-1 is involved in promoting prostate tumor growth and metastasis (Pulukuri and Rao, 2008). Therefore, the possible regulation of MMP and TIMP expression by HDAC inhibitors and the association of this regulation with the anti-invasive effect of MHY219 on prostate cancer cells were further examined. The results show that MHY219 reduced MMP-2 expres-

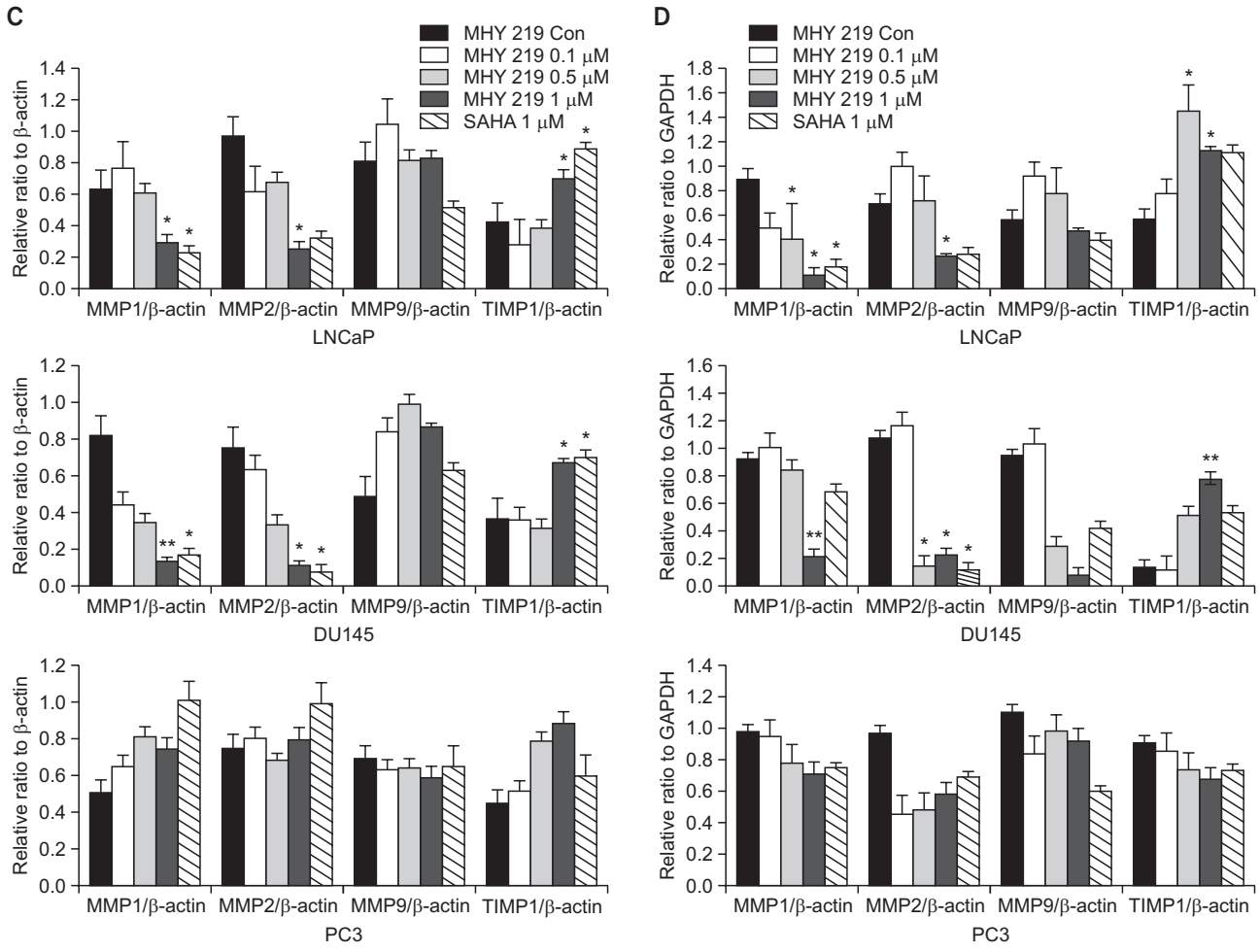


Fig. 5. Continued.

sion, whereas TIMP-1 protein and mRNA levels increased in a dose-dependent manner. The levels of MMP-1 and MMP-2, but not that of MMP-9, were significantly reduced by MHY219 treatment in LNCaP and DU145 cells, but not in PC3 cells. These results are similar to those of a previous report, which showed that apicidin significantly inhibits the H-ras-induced invasive phenotype of MCF10A human breast epithelial cells, parallel to specific downregulation of MMP-2, but not MMP-9 (Kim *et al*., 2000). The results indicate that MHY219 exerts its anti-invasive effects by reducing MMP-1 and MMP-2 expression. Uncontrolled degradation of the extracellular matrix and basement membrane are an essential part of the metastatic process (Liotta, 1986; Chambers and Matrisian, 1997). HDAC4 also plays an important role in the progression of prostate cancer from a benign to a metastatic state (Halkidou *et al*., 2004b). Treatment with HDAC inhibitors upregulates metastasis suppressor genes and downregulates metastasis promoting genes, resulting in a decrease in cancer cell invasion and metastasis (Glozak and Seto, 2007). Expression of MMPs is associated with high potential of metastasis in several human carcinomas (Pacheco *et al*., 1998; Duffy *et al*., 2000). Similarly, MHY219 significantly reduced the migration of metastatic prostate cancer cells (DU145 and PC3). In addition, MMP-1, MMP-2, and HDAC1 expression was significantly downregulated by MHY219 treatment in DU145 and PC3 cells. These result shows that MHY219 has an inhibitory effect on prostate cancer cell migration.

In conclusion, MHY219 showed anti-proliferative effects in prostate cancer cells via inhibiting HDAC1 expression. Depending on cell types, MHY219 inhibited the migration of prostate cancer cells by downregulating MMP-1 and MMP-2 expression via HDAC1 expression. These findings provide new and important information on the progression of prostate cancer. HDAC1 is closely correlated with MMP1 and MMP2 expression, supporting the role of HDAC1 in the inhibition of cancer cell migration. Additionally, as shown in this study, MMP-1 influences the metastatic potential raising the possibility that HDAC1 could be used as a molecular target in anti-metastatic therapy for patients with prostate cancer.

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