Antiproliferative effects of TSA, PXD-101 and MS-275 in A2780 and MCF7 cells: Acetylated histone H4 and acetylated tubulin as markers for HDACi potency and selectivity

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Abstract. Inhibition of histone deacetylase enzymes (HDACs) has been well documented as an attractive target for the development of chemotherapeutic drugs. The present study investigated the effects of two prototype hydroxamic acid HDAC inhibitors, namely Trichostatin A (TSA) and Belinostat (PXD-101) and the benzamide Entinostat (MS-275) in A2780 ovarian carcinoma and MCF7 breast adenocarcinoma cells. The three HDACi inhibited the proliferation of A2780 and MCF7 cells at comparable levels, below the μM range. Enzyme inhibition assays in a cell-free system showed that TSA was the most potent inhibitor of total HDAC enzyme activity followed by PXD-101 and MS-275. Incubation of A2780 and MCF7 cells with the hydroxamates TSA and PXD-101 for 24 h resulted in a dramatic increase of acetylated tubulin induction (up to 30-fold for TSA). In contrast to acetylated tubulin, western blot analysis and flow cytometry indicated that the induction of acetylated histone H4 was considerably smaller. The benzamide MS-275 exhibited nearly a 2-fold induction of acetylated histone H4 and an even smaller induction of acetylated tubulin in A2780 and MCF7 cells. Taken together, these data suggest that although the three HDACi were equipotent in inhibiting proliferation of MCF7 and A2780 cells, only the benzamide MS-275 did not induce acetylated tubulin expression, a marker of class IIb HDACs.

Introduction

Epigenetic changes have been implicated in the development of cancer through the transcriptional repression of genes that encode for key-proteins involved in regulating cellular proliferation. Mounting evidence has shown that the mechanisms that underlie these events include silencing of several tumor suppressor genes (1,2). Histone deacetylase enzymes (HDACs) comprise one of the most prominent classes of transcription factors that regulate gene expression by the removal of acetyl groups from histone and non-histone proteins (3). Deacetylation of histone proteins has been suggested to decrease the transcriptional activity of several genes as histones with less acetyl groups exhibit weaker interactions with DNA.

HDACs are broadly categorized in two families: The Zn^{+2}-dependent family that is composed of three classes of HDACs I, II and IV, and the Zn^{+2}-independent class III HDACs or SIRT enzymes (3). Class I HDACs comprise the four members HDAC1, 2, 3 and 8, which are localized in the nucleus of the cells and act on histone proteins (3). Class II HDACs are divided into the subclasses IIa comprising of HDAC 4, 5, 7, 9, and IIb comprising of HDAC6 and HDAC10 (3). Class II enzymes are primarily localized in the cytoplasm, although they are also known to shuttle in and out of the nucleus facilitating the deacetylation of several histone and non-histone proteins (2,3). Class IV includes HDAC11, whereas class III enzymes are NAD^+-dependent deacetylases with non-histone proteins as substrates in mammalian cells. Class I enzymes have been demonstrated to play a key role in cellular proliferation and survival by knockout studies, whereas class II enzymes are involved in cellular migration, differentiation and angiogenesis (3).

Due to the involvement of HDACs in the transcriptional silencing of the nuclear protein tumor suppressor genes and their implication in cellular signaling and differentiation, HDAC inhibition has emerged as a powerful tool to target cancer cells and design therapeutic drugs with improved clinical efficacy (4,5). Trichostatin A (TSA) and Belinostat (PXD-101) are two hydroxamic acid histone deacetylase inhibitors that have shown promise in the treatment of several types of cancer (6). The analogue of TSA (SAHA) used clinically, was approved for the treatment of cutaneous T-cell lymphoma (CTCL) in 2006 (7). TSA is currently undergoing multiple clinical trials in combination with other chemotherapeutic drugs, whereas PXD-101 is undergoing phase I and II trials for the treatment of various types of hematological malignancies and solid tumors such as relapsed malignant pleural mesothelioma and relapsed or refractory peripheral T-cell lymphoma (6,8-10). Entinostat (MS-275) is a benzamide-based HDACi that has been evaluated in a phase II

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study for the treatment of Hodgkin's lymphoma (11). TSA and PXD-101 have been proven to be pan-HDACi since they inhibit both class I and II enzymes, whereas MS-275 shows specificity for certain HDAC enzymes (12,13).

The present study investigated the anticancer effects of TSA, PXD-101 and MS-275 in A2780 ovarian carcinoma and MCF7 breast adenocarcinoma cells by means of total HDAC enzyme inhibition, cytotoxicity and induction of acetylated histone H4 and acetylated tubulin expression. In addition, a flow cytometric assay was employed, in order to quantify the potency of HDACi in inducing acetylated histone H4 and acetylated tubulin levels *in vitro*. The data suggest that benzamide MS-275 shows specificity towards class I enzymes as opposed to the pan-HDACi TSA and PXD-101.

Materials and methods

Reagents. MTT, DMSO, cell culture and western blot reagents were purchased from Sigma (St. Louis, MO, USA). The primary antibody for acetylated histone H4 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, USA), for acetylated tubulin from Biomol International (Plymouth Meeting, PA, USA), for HDAC1 from Abcam (Cambridge, UK), for HDAC3 from New England Biolabs-Cell Signalling (Ipswich, MA, USA) and for β-actin from Sigma. Anti-mouse and anti-rabbit secondary antibodies used for western blot analysis were from Dako (Carpinteria, CA, USA) whereas anti-rabbit secondary antibody conjugated with FITC used for flow cytometry was from Sigma.

Cell culture. A2780 and MCF7 cells were maintained in RPMI-1640 with phenol red, 2 mM glutamine, penicillin streptomycin 1X and 10% (v/v) heat-inactivated fetal calf serum. Contamination was checked by microscopic investigation. Cells were grown at 37°C, 5% CO_2 /95% air with 100% humidity, and passaged using trypsin EDTA (0.25%).

MTT cytotoxicity assay. MCF7 or A2780 ($2x10^3$) cells were plated in 96-well flat-bottomed plates. Following 24 h of incubation, the medium was removed and HDACi were added at a final concentration range of 0.039, 0.078, 0.156, 0.31, 0.625, 1.25, 2.5, 5 and 10 μM. The cells were left to grow for 96 h. The medium was removed and MTT was added in fresh medium in each well at a final concentration of 0.5 mg/ml for 3 h. The formazan product generated by viable cells was solubilised with DMSO. Cell viability was measured from the absorbance at 540 nm. Results were expressed as the percentage of 100% (control) proliferation, and the IC_{50} was calculated using Graph Pad Prism v.4.03 software.

Enzyme assay. Total HDAC activity was measured with a Fluor-de-Lys™ HDAC fluorometric activity assay kit (Biomol International). A master mix solution containing nuclear extract lysate, HDAC assay buffer, and Fluor-de-Lys™ deacetylated standard was prepared. The assay was carried out on a 96-well white microplate in the presence of HDACi at a concentration range of 20, 2, 0.2, 0.02 and 0.005 μM. The reaction was initiated by the addition of Fluor-de-Lys™ substrate, provided in the kit. Following a 20-min incubation the reaction was terminated by the addition of Fluor-de-Lys™ developer. Fluorescence was measured at an excitation λ of 360 nm and emission λ of 460 nm.

Western blot analysis. A2780 and MCF7 cells that were treated for 24 h with TSA, PXD-101 or MS-275 were trypsinised, washed once with PBS and resuspended in 100-200 μl lysis solution containing protease inhibitor cocktail and DTT (1 mM). The protein concentration of each sample was estimated by the Bradford assay, and 15 μl were mixed with SDS-PAGE 1X buffer containing 1.5 ml of Tris base (1 M pH 6.8), 2.5 ml of 20% SDS, 2.5 ml of 100% glycerol, bromophenol blue and β-mercaptoethanol at a concentration of 0.1% (w/v) and 10% (v/v), respectively. The running and stacking gel (10%) were composed of 7.9 and 2.5 ml of H_2O, 6.7 and 0.625 ml of 30% acrylamide, 5 and 1.05 ml of 1.5 M Tris·Cl (pH 8.8), 0.2 and 0.04 ml of 10% sodium dodecyl sulfate (SDS), 0.2 and 0.02 ml of 10% ammonium persulfate (APS) and 9 μl of TEMED (*N-,N-* tetramethylethylenediamine), respectively. The running buffer was prepared as 5X stock by mixing 75.5 g of Tris, 360 g of glycine and 25 g of SDS with 5 l of H_2O. The gel was left to run for 1 h at 120 V and the proteins were transferred to a PVDF membrane at a constant current of 300 mA for 1 h. The membrane was removed and incubated with 5% milk in TBS-T at room temperature for 1.5 h with gentle shaking or overnight at 4˚C, depending on the experiment. The membrane was then incubated either overnight at 4˚C or at room temperature for 1.5-3 h in 1% milk in TBS-T, containing primary antibody. The following morning, the membrane was rinsed briefly with TBS-T and washed three times with TBS-T for 10 min. Secondary antibody was diluted in 1% milk TBS-T and added to the membrane for 1 h at room temperature with gentle shaking. The membrane was then washed three times for 10 min with TBS-T and incubated with 1.5-2 ml of ECL Plus detection reagents for 5 min at room temperature. The membrane was finally exposed for 2-30 min to a film and developed using a standard developer and fixer solutions.

The primary antibodies used were as follows: Acetylated histone H4 for 3 h at 1:20,000 dilution, acetylated tubulin for 1.5-2 h at 1:6,000 dilution, HDAC1 for overnight at 1:500 dilution, HDAC3 for overnight at 1:1,000 dilution and β-actin for 1 h at 1:10,000 dilution. The secondary antibodies used were the following: Anti-mouse IgG for 1 h at 1:1,000 dilution and anti-rabbit IgG for 1 h at 1:2,000 dilution.

Flow cytometry. The method was adapted from Ronzoni *et al* (14). Briefly, A2780 and/or MCF7 cells were seeded at a density of $5x10^3$ cells/ml and left to grow for 24 h. HDACi were added at a final concentration of 2, 5 and/or 10 μM and incubated with the cells for another 24 h. The cells were washed with PBS once, detached from the flasks with the aid of trypsin-EDTA and resuspended in ice-cold PBS containing 1% formalin. Following incubation on ice for 15 min the cells were centrifuged at 3,500 rpm for 5 min and resuspended in 70% ice-cold ethanol. The same process was conducted and the cells were finally resuspended in PBS containing 0.1% Triton-X. The supernatant was removed and 1% BSA in PBS was added to each sample that was vortexed, incubated at room temperature for 15 min and centrifuged at 3,500 rpm for 5 min. The supernatant was discarded and blocking of the

non-specific binding sites was achieved by the addition of PBS containing 10% normal goat serum and incubation on a rocker for 20 min at room temperature. A primary antibody of acetylated histone H4 and/or acetylated tubulin was added at a 1:100 or 1:200 dilution, respectively, in PBS containing 1% BSA and incubated with the cells for 1 h at room temperature by continuous shaking. The cells were washed once with PBS. Secondary antibody conjugated with FITC was added in PBS 1% BSA at a 1:1,000 dilution and incubated with the cells in the dark for 1 h at room temperature by continuous shaking. The cells were finally centrifuged at 3,500 rpm for 5 min and the supernatant was discarded. PI (50 μg/ml) and RNAse A (10 μg/ml) were added to the samples that were incubated in the dark for 30 min. The fluorescence intensity was measured using a BD FACSCalibur flow cytometer with an excitation λ of 488 nm and emission λ of 520 nm for FITC, and 625 nm for PI. A total of 3 controls were prepared: One containing no stain, one with PI alone and one with FITC alone.

Statistical analysis. The results are expressed as mean ± SD for n=3 determinations unless indicated otherwise. Statistical differences were determined with a paired t-test.

Results

HDACi inhibit proliferation of A2780 and MCF7 cells. HDACs have been validated as targets for anticancer therapy. The inhibitors TSA, PXD-101 and MS-275 (Fig. 1) were the initial small molecules designed to test the therapeutic potential of HDAC enzymes and have shown promise in the treatment of solid tumors and hematological cancers (6,8,9). It has been reported that among different tumor types, breast and ovarian cancers are responsive to HDACi treatment (15,16). Thus, the antiproliferative effects of TSA, PXD-101 and MS-275 were examined in A2780 ovarian carcinoma and MCF7 breast adenocarcinoma cells by the MTT cell viability assay. All HDACi exhibited comparable IC_{50}s, below the μM scale (Table I). PXD-101 showed a somewhat greater potency in A2780 cells compared with MS-275 and TSA, whereas in MCF7 cells, MS-275 was the most effective inhibitor of cellular proliferation. The PI staining of A2780 cells showed that both PXD-101 and TSA caused a blockage at the G2/M phase of the cell cycle at 5 and 10 μM with a combined induction of apoptosis, compared to control samples treated with 0.1% DMSO for 24 h (Fig. 2A and B). In contrast to TSA and PXD-101, MS-275 produced a G1 block in A2780 cells compared with the control sample (Fig. 2A and B). It is important to note that TSA induced higher G2/M arrest (83±2.4%) compared with PXD-101 (78±1.9%) in A2780 cells (Fig. 2B).

The hydroxamic acids PXD-101 and TSA exhibit higher potency with regard to HDAC inhibition than MS-275 in enzyme and cell-based assays. In an effort to examine the association of the antiproliferative effect with HDAC enzyme inhibition, the ability of HDACi to inhibit total HDAC enzyme activity was further investigated in a cell-free assay system that utilizes a fluorogenic acetylated lysine side chain as a substrate. TSA was the most potent inhibitor with an IC_{50} lower than 0.01 μM, whereas MS-275 was considerably weaker with an IC_{50} of 2 μM (Fig. 2C). PXD-101 indicated intermediate efficacy with regard to HDAC enzyme inhibition, exhibiting an IC_{50} of 0.04 μM (Fig. 2C).

To extend the relevancy of the cell-free enzyme inhibition results, the effect of HDACi on the induction of acetylated histone H4 and acetylated tubulin was examined in MCF7 and A2780 cells. Acetylated histone H4 is a marker of HDAC1 and HDAC3 activity, which were constitutively expressed in A2780 and MCF7 cells (Fig. 3A). Western blot analysis clearly demonstrated that both MS-275 and TSA induced a high increase in the expression levels of acetylated histone H4 in A2780 cells compared with the solvent control (0.1% DMSO) (Fig. 3B). Using immunoblotting, the potency of these two inhibitors was initially found to be very similar. Consequently, a flow cytometry assay was employed to quantify the increase of acetylated histone H4, following HDACi treatment (Fig. 3B). The methodology involved incubation of the samples with high concentrations of primary antibody (1:100 dilution) and detection using a secondary antibody conjugated to FITC as described by Ronzoni *et al* (14). The linearity of the assay was confirmed by treatment of A2780

Table I. Antiproliferative activity of MS-275, TSA and PXD-101 in A2780 and MCF7 cells.

Compounds	A2780 (μM)	MCF7 (μM)
TSA	0.5±0	0.6±0.01
PXD-101	0.4±0.05	0.4±0
MS-275	0.6±0	0.4±0.04

Antiproliferative activity of MS-275, TSA and PXD-101 in A2780 and MCF7 cells were measured as described in Materials and methods. The values indicate the mean ± SD of the IC_{50} for at least n=3 determinations. TSA, Trichostatin A; PXD-101, Belinostat; MS-275, Entinostat; SD, standard deviation.

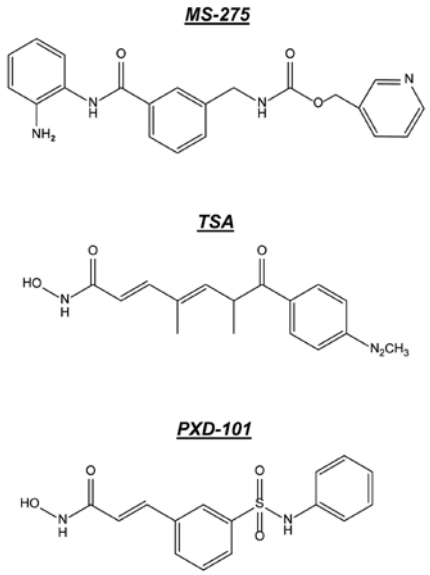

Figure 1. Chemical structures of MS-275, TSA and PXD-101. MS-275, Entinostat; TSA, Trichostatin A; PXD-101, Belinostat.

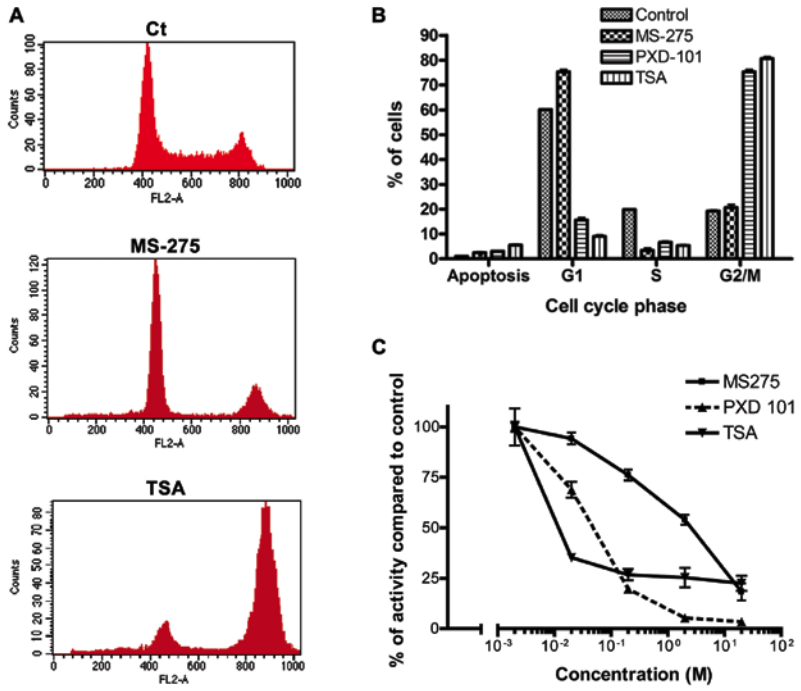

Figure 2. Anticancer effects of the HDACi MS-275, PXD-101 and TSA. (A) Cell cycle analysis of A2780 cells that were treated for 24 h with 5 μM of MS-275 and/or TSA. (B) Proportion of cells in each phase was measured with PI staining. (C) Total HDAC enzyme inhibition of MS-275, PXD-101 and TSA. Enzyme activity assay was performed using a Biomol kit and a concentration range of 20, 2, 0.2, 0.02 and 0.005 μM of each inhibitor as described in Materials and methods. HDACi, histone deacetylase enzyme inhibitor; MS-275, Entinostat; PXD-101, Belinostat; TSA, Trichostatin A.

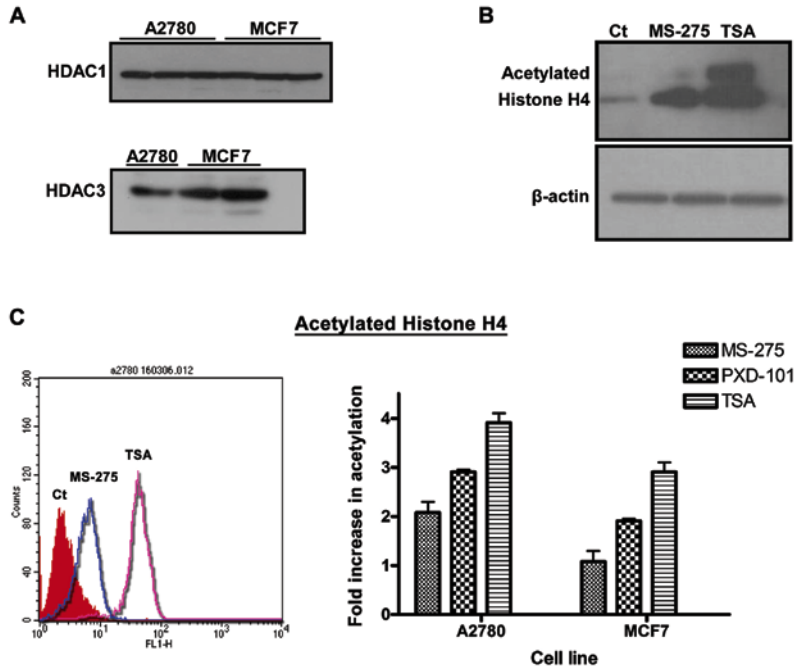

Figure 3. HDACi-induced acetylated histone H4 expression in A2780 and MCF7 cells. (A) Expression profile of HDAC1 and HDAC3 in A2780 and MCF7 cells. Cells were probed with primary anti-HDAC1 and anti-HDAC3 antibodies and the protein expression profile was developed by immunoblotting using ECL reagents as determined in Materials and methods. HDAC1; lanes 1-3: A2780 cells, lanes 4-6: MCF7 cells, HDAC3; lane 1: A2780 cells, lanes 2-3: MCF7 cells. (B) Upregulation of acetylated histone H4 expression in A2780 cells following HDACi treatment was monitored by western blot analysis and flow cytometry as described in Materials and methods. A2780 cells were treated with 0.1% DMSO, 10 μM MS-275 and 5 μM TSA for 24 h. (C) Fold increase of acetylated histone H4 as determined by flow cytometry following a 24-h treatment of 5 μM of HDACi. Left: Flow cytometry histogram, Right: Relative quantification of fluorescence signal. The error bars represent SD for at least n=3 determinations. HDACi, histone deacetylase enzyme inhibitor; TSA, Trichostatin A; SD, standard deviation.

and/or MCF7 cells with known concentrations of HDACi (0, 2, 5 and 10 μM) (data not shown). MS-275 induced a 2-fold increase in acetylated histone H4, whereas PXD-101 and TSA were more potent inducing a 3- and 4-fold increase in A2780 cells, respectively (Fig. 3C). Similar results were obtained in MCF7 cells for the three HDACi (Fig. 3C).

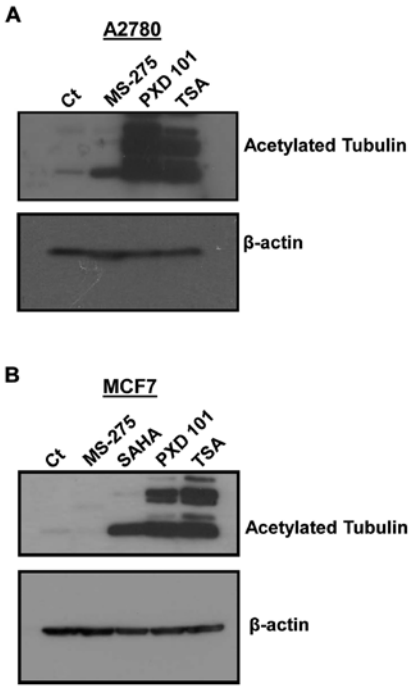

Figure 4. HDACi-increased expression of acetylated tubulin in A2780 and MCF7 cells. A2780 and MCF7 cells were treated with 5 μM HDACi for 24 h and acetylated tubulin was measured by western blot analysis, as described in Materials and methods. (A) A2780 left to right lane 1-4: Control (0.1% DMSO), MS-275, PXD-101, TSA. (B) MCF7 left to right lane 1-5: Control (0.1% DMSO), MS-275, SAHA, PXD-101, TSA. HDACi, histone deacetylase enzyme inhibitor; MS-275, Entinostat; PXD-101, Belinostat; TSA, Trichostatin A.

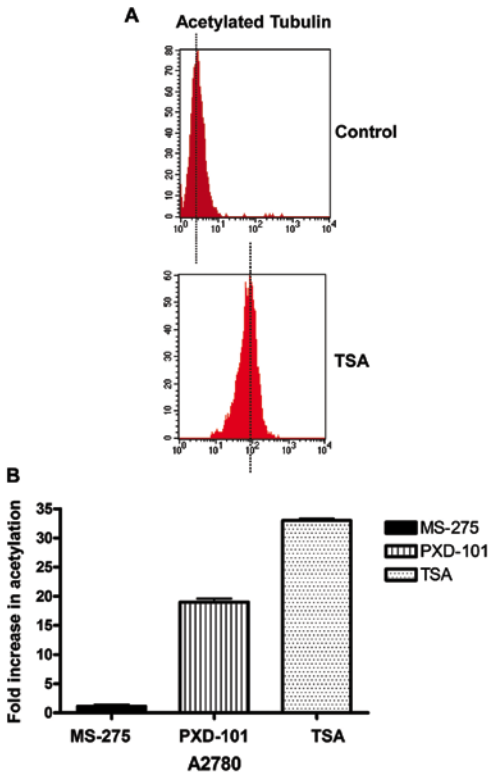

Figure 5. Measurement of TSA-mediated acetylated tubulin induction in A2780 cells by flow cytometry. (A) Flow histogram of A2780 cells treated with 5 μM TSA for 24 h that were probed with anti-acetylated tubulin primary antibody and secondary anti-IgG conjugated with FITC. (B) Fold increase in acetylated tubulin induction of A2780 cells following 5 μM treatment of HDACi for 24 h. The results indicate the mean ± SD for n=3 determinations. TSA, Trichostatin A; HDACi, histone deacetylase enzyme inhibitor; SD, standard deviation.

PXD-101 and TSA induce potent upregulation of acetylated tubulin compared with MS-275 in A2780 and MCF7 cells. The effects of HDACi on the induction of acetylated tubulin expression, which is a marker of HDAC6 enzyme activity, were markedly different to those obtained for acetylated histone H4 in A2780. MS-275 induced a very weak increase of acetylated tubulin expression in A2780 cells, whereas PXD-101 and TSA were considerably more potent, as determined by western blot analysis (Fig. 4A and B). Moreover, the flow cytometry analysis demonstrated that the fold-increase in the induction of acetylated tubulin caused by 5 μM of MS-275 in A2780 cells was negligible, compared to the other two HDACi, where a remarkable 18- and 30-fold increase was observed (Fig. 5A and B).

Discussion

Induction of acetylated histone H4 is a common end-result observed following HDACi treatment. This protein has been proposed as a marker for the diagnosis and evaluation of HDACi efficacy in clinical trials involving human solid tumors (17). Flow cytometry was previously employed and has successfully been validated as a powerful tool for the detection of acetylated histone H4 levels in blood samples from patients, as well as leukemic cell lines that were treated with HDACi such as valproic acid and TSA (14,18). The results presented in the current study indicated that TSA was the most effective inducer of acetylated histone H4, compared to the other two HDACi. Using western blot analysis, Duong *et al* (19) reported similar findings. A higher induction in the levels of acetylated histone H4 was noted in MCF7 cells treated with TSA compared with cells that were treated with MS-275 (19). Ronzoni *et al* (14) demonstrated a 4-fold induction of acetylated histone H4 in U937 leukemic cells treated with 50 ng/ml TSA for 4 h, which corresponded to an approximate concentration of 0.2 μM. This increase was similar to that noted in the present study, although the concentration and incubation times used were considerably higher. Despite this discrepancy, the maximum induction in the study conducted in U937 cells was noted at the 4-h period. It is important to note that U937 leukemic cells may be more sensitive to HDACi treatment than either MCF7 or A2780 cells, thereby accounting for the difference in the concentration of TSA, required for maximum induction.

A previous study by Khan *et al* (12) reported on the class and isoform selectivity of small molecule HDAC inhibitors. The authors used a similar enzymatic assay to the one described in the present study and recombinant human HDAC isoforms to determine the potency of each inhibitor. MS-275 was shown to be selective for HDAC1, whereas both TSA and PXD-101 were potent pan-HDAC inhibitors, although both classes of inhibitors inhibited HeLa cell growth. In the present study, TSA and PXD-101 exhibited a higher potency than MS-275 in inhibiting HDAC enzyme activity. One possible explanation is that the Fluor-de-Lys™ enzyme assay utilizes

a HeLa nuclear lysate, which contains all HDAC isoforms, rather than recombinant HDAC enzymes, thus, accounting for the IC_{50} difference noted between the hydroxamic acid HDACi and MS-275.

Previous reports have underlined the antitumor effect of HDACi in cancer cell line models. MS-275, PXD-101 and TSA show considerably low IC_{50}s, below the μM range (15,20-22). PXD-101 has been shown to inhibit proliferation of A2780 cells at a higher potency than MCF7, with IC_{50} values of 30 and 50 nM, respectively. In contrast to the study by Qian *et al* (22), TSA exhibited a 90% reduction of cellular proliferation in A2780 cells at 100 ng/ml following a 3-day incubation, which corresponded to an approximate IC_{50} value of 0.8 μM (23-25). Duong *et al* (19) previously reported that, in MCF7 cells, TSA exhibited approximately 75% reduction of proliferation at 0.07 μM following a 2-day treatment and 85% following a 5-day treatment. This corresponds to approximate IC_{50}s of 0.15 and 0.25 μM, whereas Davies *et al* (15) showed a 50% reduction of MCF7 cell growth caused by treatment of 1 μM TSA for 48 h. These published data are in agreement with the results presented in the current study. The mechanism of action of hydroxamic acid HDACi involves cell cycle arrest at the G2/M phase through p21 upregulation and induction of apoptosis via Bcl-2 expression (19,23-25).

Using western blot analysis, Duong *et al* (19) reported on the potent induction of acetylated tubulin in MCF7 cells treated with 1.7 μM of TSA for 6 h, while treatment of 1 μM of MS-275 for the same time period had no effect on the expression of the latter protein, which concurs with our findings. In A2780 cells, acetylated tubulin was upregulated following a 24-h treatment of TSA and/or PXD-101 at a concentration range of 0.3, 1 and 3 μM, as opposed to MS-275 where the levels of protein expression remained constant and similar to the control sample (23). In concordance with the studies by Duong *et al* (19) and Arts *et al* (23), we demonstrated upregulation of acetylated tubulin following HDACi treatment in MCF7 cells by western blot analysis, and in A2780 cells by flow cytometry and western blot analysis. FACS has been used as a method to detect acetylated histone H4 in cell lines and clinical samples (14). To the best of our knowledge, acetylated tubulin induction following HDACi treatment has only been detected by immunoblotting. Application of the flow cytometry protocol described previously for acetylated histone H4 expression showed that the induction of acetylated tubulin was higher by a factor of 10, when the cells were incubated with either TSA and/or PXD-101. It is noteworthy that incubation of either PXD-101 and/or TSA with MCF7 and/or A2780 cells, produced a number of bands corresponding to multiple levels of tubulin acetylation, compared with MS-275 where a similar expression to the control was noted (Fig. 4A and B). In contrast to these observations, acetylated histone H4 induction was evident by the presence of two bands, corresponding to two levels of acetylation (Fig. 3B). Since acetylated tubulin induction was a more sensitive marker of hydroxamic acid HDACi treatment, compared with acetylated histone H4, the western blot analysis results are in concordance with the flow cytometry analysis undertaken in the present study. The data confirm that TSA and PXD-101 are pan-HDACi, whereas MS-275 does not inhibit some of the class II enzyme isoforms such as HDAC6.

Investigation of the mechanisms and function of HDACs in tumor progression is an active research area that has attracted considerable scientific attention in recent years. Although the exact molecular pathways by which HDAC enzymes contribute to cancer progression remain ill-defined, it is generally believed that class I HDACs play a significant role in cellular proliferation, whereas class II enzymes are involved in other processes such as angiogenesis, adhesion and differentiation (2). It is becoming increasingly evident that targeting class I enzymes is more beneficial in cancer therapy due to the pleiotropic effects of HDACi in multiple cellular signaling pathways such as induction of apoptosis and induction of cell cycle inhibition (26). In addition, the design of class- or HDAC-specific small molecule inhibitors, such as MS-275, is essential in order to unravel the mechanism of action of each HDAC enzyme, since HDACs are known to participate in large protein complexes and interact with several important transcriptional factors that regulate cell growth, remodeling and differentiation, namely p300 and Snail (27,28).

The present study therefore demonstrated the selectivity and potency of three well-known HDACi in *in vitro* cell and enzyme assays. The data demonstrated that MS-275 is a more selective inhibitor of HDACs than either TSA or PXD-101, while all compounds indicated comparable submicromolar IC_{50}s against A2780 and MCF7 cells. Future investigations should focus on the design of novel class I specific benzamide-based HDACi as anticancer agents.

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