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
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 HDACi 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% CO2/95% 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 1.25, 2.5, 5 and 10 µM. The cells were left to grow for 96 h. The bation, the medium was removed and HDACi were added at a final 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™ substrate, provided in the kit. Following a 20-min incubation the reaction was terminated by the addition of Fluor-de-Lys™ substrate, provided in the kit.

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™ substrate, provided in the kit. Following a 20-min incubation the reaction was terminated 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™ substrate, provided in the kit. Following a 20-min incubation the reaction was terminated 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™ substrate, provided in the kit.
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 IC50s, 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 IC50 lower than 0.01 µM, whereas MS-275 was considerably weaker with an IC50 of 2 µM (Fig. 2C). PXD-101 indicated intermediate efficacy with regard to HDAC enzyme inhibition, exhibiting an IC50 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 by the MTT cell viability assay. All HDACi exhibited comparable IC50s, 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).

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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 cells with two concentrations of primary antibody (1:100 and 1:200 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 cells with two concentrations of primary antibody (1:100 and 1:200 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 cells with two concentrations of primary antibody (1:100 and 1:200 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 cells with two concentrations of primary antibody (1:100 and 1:200 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 cells with two concentrations of primary antibody (1:100 and 1:200 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 cells with two concentrations of primary antibody (1:100 and 1:200 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 cells with two concentrations of primary antibody (1:100 and 1:200 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 cells with two concentrations of primary antibody (1:100 and 1:200 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 ce
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).
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 IC₅₀ 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 IC₅₀, 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 IC₅₀ 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 IC₅₀ 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 IC₅₀ 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 IC₅₀ 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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