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## Determination of the class and isoform selectivity of small molecule HDAC inhibitors

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Short Running title: HDAC inhibitors: class and isoform selectivity

### Abstract

The human histone deacetylase (HDAC) family, a well-validated anti-cancer target, plays a key role in the control of gene expression through regulation of transcription. While HDACs can be subdivided into three main classes, the class I, class II and class III HDACs (Sirtuins), it is presently unclear whether inhibiting multiple HDACs using pan HDAC inhibitors, or targeting specific isoforms that show aberrant levels in tumors, will prove more effective as an anti-cancer strategy in the clinic.

To address the above issues, we have tested a number of clinically relevant HDAC inhibitors (HDACi) against a panel of recombinant human HDAC (rhHDAC) isoforms. Eight rhHDACs were expressed using a baculoviral system, and a Fluor de Lys<sup>TM</sup> (FDL) HDAC assay was optimized for each purified isoform. The potency and selectivity of ten HDAC inhibitors on class I isoforms (rhHDAC1, 2, 3 and 8) and class II HDAC isoforms (rhHDAC 4, 6, 7 and 9) was determined. MS-275 was HDAC1 selective, MGCD0103 was HDAC1 and HDAC2 selective, apicidin was HDAC2 and HDAC3 selective and valproic acid was an HDAC class I specific

inhibitor. The hydroxamic acid-derived compounds (TSA, NVP-LAQ824, panobinostat, ITF2357, vorinostat and belinostat) were potent pan HDAC inhibitors. The growth inhibitory effect of the HDACi on HeLa cells showed that both pan HDAC and class I specific inhibitors inhibited cell growth. The data also showed that both pan HDAC and class I specific inhibitor treatment resulted in increased acetylation of histones, but only the pan HDAC inhibitors resulted in increased tubulin acetylation, which is in agreement with their activity on the HDAC6 isoform.

**Key Words:** HDAC, HDAC isoform, inhibitors, deacetylation, tubulin

**Abbreviations used:** HDACs, Histone deacetylases; SMRT, silencing mediator for retinoid and thyroid receptors; NCoR, nuclear receptor corepressor; DAD, deacetylase activating domain; HDACi, HDAC inhibitor; valproic acid, VPA

## **Introduction**

Histone deacetylases (HDACs) are a family of enzymes involved in the regulation of a number of cellular processes [1], including cell proliferation, apoptosis and assembly of the cytoskeleton. However, the best characterized HDAC function is the control of gene expression through regulation of transcription [2, 3]. This involves an interplay between histone acetyltransferases (HATs) and HDACs, both of which are involved in post-translational modification of histone proteins [4]. HATs and HDACs have opposing roles in acetylating and deacetylating highly conserved lysine residues on the N-terminal tail of histones, thus altering chromatin assembly and transcriptional activity [5, 6]. HDACs are also involved in regulating the acetylation of a number of non-histone proteins, such as  $\alpha$ -tubulin and p53 [7, 8, 9]. These data, and reports of aberrant HDAC activity in a number of tumor types [10], suggest that inhibition of HDACs represents a viable anti-cancer strategy [11, 12, 13].

To date, 18 members of the human histone deacetylase family have been identified. These HDACs are subdivided into three individual classes based on structural and

functional similarities. The class I isoforms (HDACs 1, 2, 3 and 8) and class II (HDACs 4, 5, 6, 7, 9, 10 and 11) are Zn-dependent enzymes, whereas class III HDACs (Sirtuins (Sir1-7)), are NAD-dependent [14, 15]. HDACs exist in large multiprotein complexes, and there is evidence that most, if not all, HDAC isoforms require interaction with other HDACs or proteins for optimal enzymatic activity. For example, HDAC3 activity requires an interaction with SMRT/NCOR [16, 17], whereas suppression of HDAC4 binding by the HDAC3 - SMRT/NCOR complex results in the loss of HDAC4 enzymatic activity [18]. More recently, the presence of two intramolecular or intermolecular catalytic domains was reported to be required for protein deacetylation [19].

A number of HDAC inhibitors are currently undergoing clinical trials as anti-cancer drugs [20, 21, 22], some of which have been characterized as selective HDAC inhibitors, and some as pan HDAC inhibitors. Recently vorinostat was registered as an HDACi drug (Zolinza). In the present study, we provide a comprehensive evaluation of the potency and selectivity of various HDAC inhibitors versus recombinant HDAC isoforms.

## Materials and Methods

Belinostat, MS-275, vorinostat, NVP-LAQ824, panobinostat, ITF2357 and MGCD0103 were synthesized as described in recent patent applications (International publication number - belinostat: US6888027, MS-275: US6174905, vorinostat: US5369108, ITF2357: WO03/013493, NVP-LAQ824: US6552065, panobinostat: US6552065 and MGCD0103: US6897220). All solvents were purified before use by routine techniques. The reaction products were isolated by evaporating the solvent (vacuum rotary evaporator). Compounds were purified using column chromatography on a silicagel, 0.035-0.070 mm column (Acros). Reversed phase HPLC on a Varian ProStar HPLC system, equipped with a spectrophotometer; was used to check purity of the synthesized compounds. The melting point was determined with micro melting point apparatus (Boëtius or Fisher) and the uncorrected values were used. NMR spectra, obtained on Varian WH-90/DS or Mercury 200 spectrometers, were used to

confirm the chemical structure of the compounds. The chemical shift ( $\delta$ ) values are presented in parts per million (ppm). Elemental analyses were carried out on a Carlo Erba EA 1108 instrument [23]. Apicidin, TSA and valproic acid were purchased from Sigma-Aldrich.

### **Expression of HDAC isoforms**

Recombinant HDAC isoforms were produced using the Baculovirus Expression Vector System. In addition, mouse silencing mediator for retinoid and thyroid receptors (SMRT), a co-activator of HDAC 3 was also expressed. The deacetylase activating domain (DAD) of SMRT, constituted by 95 amino acids (395-489 in mouse SMRT) was expressed with a translation initiation codon (methionine) added to the N-terminus.

### **Cloning of cDNAs**

Complementary DNAs comprising the complete coding sequences of the human HDAC isoforms and the DAD portion of mouse SMRT were isolated from a human cDNA library by PCRs using primers listed in Supplementary Table 1. Restriction sites for either BamH I or Bgl II were incorporated in the sense primers, and Xho I or Sal I in the antisense primers in order to facilitate subsequent cloning. In addition, sequences coding for either Flag or 6xHis tags were incorporated in the primers. The PCR amplicons were subcloned into the cloning vector pCR2.1TOPO and nucleotide sequences were determined. The cDNAs were then subcloned into BamH I and Xho I sites of appropriate expression vectors (Supplementary Table 2).

### **Generation of recombinant baculoviruses**

Recombinant baculoviruses were generated using either the Bac-N-Blue™ Baculovirus System or the Bac-to-Bac® Baculovirus Expression System according to the instructions of the manufacturer (Invitrogen, Carlsbad, CA; Supplementary Table 2). *Spodoptera frugiperda* - derived Sf9 cells cultured in Ex-Cell 420 medium supplemented with 5% FBS served as host cells for virus generation and/or protein production.

The Bac-N-Blue™ Baculovirus System was used to produce recombinant HDAC isoforms with no fusion protein; however Flag tags were added to the C-termini. In this system the cDNAs were first subcloned into the transfer vector pBlueBac4.5. Sf9 cells grown in adherent format were co-transfected with the transfer vector carrying the HDAC coding sequence and Bac-N-Blue™ linearized baculovirus DNA. Recombinant viruses were collected and cloned in plaque assays. Six to ten virus clones were tested in a small scale infection for their ability to produce recombinant HDAC and to generate low titer virus stock. One of these virus stocks was selected for generating high-titer virus stock and large-scale infection. High-titer virus stocks were produced by infecting a 200-ml culture of Sf9 cells ( $1.5 - 2.0 \times 10^6$  cells/ml) with 200  $\mu$ l of the low-titer stock. Virus-laden media were harvested when the cell viabilities dropped to 40 % or below (4-6 days post infection). Titer of the viral stocks was determined using the FastPlax™ Titer Kit (Novagen, San Diego, CA).

HDAC isoforms with N-terminal fusion of Glutathione-S-Transferase (GST) with or without Flag tags were produced using the Bac-to-Bac® Baculovirus Expression System. The transfer vector pFastBac1 was first modified to include the GST coding sequence at the 5' end of the multiple cloning site. The HDAC cDNAs were inserted downstream of the GST coding sequence through subcloning. Competent *E. coli* DH10Bac cells were transformed with the recombinant transfer vector. Recombinant bacmids were isolated and verified for the presence of the cDNAs coding for HDACs by PCR, using appropriate primers. Sf9 cells were transfected with the bacmid, and the resulting recombinant viruses were tested for their ability to produce recombinant HDACs. This low-titer stock was used to generate a larger scale and high-titer viral stock as described above.

### **Large-scale infections**

Production cultures were performed either in two to four 3-L shake flasks (1 L culture volume per flask) or in 20-L wave bioreactors (10 L culture volume, Somerset, NJ). Flasks or wave bioreactors were seeded with Sf9 cells at  $0.5 \times 10^6$  cells/ml. When the cell densities reached  $1.5 - 2.0 \times 10^6$  cells/ml, the cultures were inoculated with

appropriate recombinant baculovirus stocks at multiplicities of infection of 3 to 5. Three days post infection; cells were harvested by centrifugation at 1200 X g for 15 min at 4 °C in a Sorvall Evolution RC centrifuge (New Town, CT). Cell pellets were stored at -80 °C until protein purification.

### **Purification of recombinant HDAC isoforms**

#### **Lysis of infected cell pellets**

Infected cells were resuspended in ice-cold lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 1 % Triton-X-100, pH 8.0), using 1 ml of lysis buffer for every  $2 \times 10^7$  cells. Complete EDTA-free protease inhibitor cocktail tablets (Roche Applied Science, Indianapolis IN) were dissolved in the lysis buffer just prior to use at a ratio of one tablet for every 50 ml of buffer. The suspension was homogenized for 1 min at a setting of 1 on a T 25 basic ULTRA-TURRAX dispersing instrument (IKA Works, Wilmington NC) and incubated on ice for 45 minutes. Insoluble material was removed by centrifugation at 23,000 X g at 4 °C for 45 minutes in a Sorvall Evolution RC centrifuge (Newtown CT). The clarified lysate was processed immediately by batch chromatography.

#### **Batch-mode affinity purification using glutathione sepharose**

GST-tagged proteins were purified using Glutathione Sepharose 4 Fast Flow (GE, Piscataway NJ) (Supplementary Table 2). Settled Sepharose beads were washed by suspending in a 10-fold excess of ice-cold lysis buffer followed by centrifugation at 4 °C for 5 minutes at 213 X g in a Sorvall Legend RT tabletop centrifuge equipped with a swinging-bucket rotor. The wash step was repeated two more times. The washed beads were suspended in lysis buffer to make a 50 % slurry. The lysate was split into 50-ml aliquots in disposable centrifuge tubes, and 1.25 ml of Sepharose slurry was added to each tube. Tubes were incubated for one hour at 4 °C with end-over-end mixing. The charged matrix was collected by centrifugation, and the beads were washed by centrifugation three times with 40 ml of ice-cold PBS. Finally, the matrix was resuspended in PBS and transferred to a Poly-Prep disposable chromatography column (Bio-Rad Laboratories, Hercules CA). The column was placed at 4 °C and

allowed to drain until the top of the bed was dry. One-third packed bed volume of ice-cold elution buffer (50 mM HEPES, 40 mM reduced L-glutathione, pH 9.0) was carefully added to the top of the bed and allowed to drain through the column. The effluent was discarded. Two packed bed volumes of elution buffer were carefully added to the top of the bed, and the effluent was collected as a single fraction. The effluent containing the HDAC was immediately aliquoted and frozen at  $-80^{\circ}\text{C}$ .

### **Batch-mode affinity purification using anti-FLAG agarose**

FLAG-tagged proteins were purified using ANTI-FLAG M2 Affinity Gel (Sigma, St. Louis MO) (Supplementary Table 2). Settled agarose beads were washed as described above, twice with ice-cold PBS, once with 0.1 M glycine, pH 2.8, and twice with PBS containing an additional 150 mM NaCl. The washed beads were suspended in PBS containing an additional 150 mM NaCl to make a 50 % slurry. Batch-mode capture, washing with PBS, and transfer to a Poly-Prep chromatography column were performed as described above. The column was placed at  $4^{\circ}\text{C}$  and allowed to drain until the top of the bed was dry. Elution buffer was prepared as follows. 3X FLAG peptide (Sigma, St. Louis MO) was dissolved at a concentration of 25  $\mu\text{g/ml}$  in 0.5 M Tris-HCl, 1 M NaCl, pH 7.5 and the solution was diluted 1:4 with water to make a 5  $\mu\text{g/ml}$  stock which was stored at  $-20^{\circ}\text{C}$ . Elution buffer was prepared just prior to use by adding the 3 X FLAG peptide stock to PBS to a final concentration of 250 ng/ml. The pre-elution and elution steps, and sample aliquoting were performed as described above.

### **HDAC assays**

Deacetylase activity of rhHDACs1, 2, 3, 4, 6, 7 and 9 was assayed with a pan HDAC substrate (initially KI-104, Biomol and subsequently I-1925, Bachem). Deacetylase activity of rhHDAC8 was assayed with HDAC8 specific Fluor de Lys<sup>TM</sup> (FDL) substrate (KI-178, Biomol). The total pan HDAC assay volume was 50  $\mu\text{l}$  and all the assay components were diluted in HEPES buffer (25 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 4.9 mM  $\text{MgCl}_2$ , pH. 8.0). The reaction was carried out in half volume white 96-well plates (Costar 3693). In brief, the pan HDAC assay contained HDAC



substrate (30  $\mu$ M, 25  $\mu$ l); rhHDAC isoform (0.5 - 3  $\mu$ l, diluted to 15  $\mu$ l final volume) and inhibitor (5 X the required assay concentration, diluted to 10  $\mu$ l final volume). Positive controls contained all the above components, except the inhibitor. The negative controls did not contain either enzyme or inhibitor. In each case these were replaced with an equivalent volume of buffer. The assay components were incubated at 37°C for 3 h. The reaction was quenched with the addition of 50  $\mu$ l HDAC-FDL Developer (KI-105, Biomol) 20 X stock diluted to 1:400 in HEPES buffer and containing 2  $\mu$ M TSA. The plates were incubated (25 min) at room temperature to allow the fluorescent signal to develop. The fluorescence generated was monitored at 355/460 nm (excitation/emission) wavelength.

The HDAC8 specific assay was as described for the pan HDAC assay, except for the changes indicated. The HEPES assay buffer contained BSA (1 mg/ml). The FDL-HDAC8 substrate was 25  $\mu$ M. The assay components were incubated (3 h) at room temperature and then the reaction was quenched with HDAC-FDL Developer (KI-176, Biomol) 5 X stock diluted 1:110 in HEPES buffer containing BSA (1mg/ml) and 2  $\mu$ M TSA. The plates were incubated (25 min) at 4°C to allow the fluorescent signal to develop.

### **Cell proliferation assays**

HeLa cells were grown in monoculture and treated with serial dilutions of the HDACi. The cells were incubated (48 h) at 37°C. The number of viable cells was assessed using Cell Proliferation Reagent WST-1. Media only and HeLa cells without HDACi were used as negative and positive controls, respectively.

### **Immunoblotting**

Cells grown in monolayer culture were treated with compounds as indicated in the figure legends. Cells were harvested in 2 X Tris-glycine SDS sample buffer (Invitrogen) supplemented with 10 mM DTT (Sigma). Cell lysates were boiled for 10 minutes, resolved on 4-20% gradient SDS-polyacrylamide gels (Invitrogen), and transferred to nitrocellulose membranes (Invitrogen). Immunoblotting was performed using standard procedures and the following primary antibodies: anti-acetylated

(lys40) alpha-tubulin (Sigma, T6793), anti-acetylated (lys9) histone H3 (Cell Signaling Technology, 9671), anti-acetylated lysine (Ac-K-103, Cell Signaling Technology, 9681), anti-p53 (mix of antibodies from Santa Cruz Biotechnology, sc-126 and Calbiochem, OP03) and anti-actin (Sigma, A5060). Following incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies, enhanced chemiluminescence (GE Healthcare) was used for detection.

## Results

### Cloning and Expression

Eight full length recombinant human HDACs (rhHDAC) were expressed in insect cells using a baculoviral expression system. The purified rhHDACs consisted of four class I enzymes (FLAG-HDAC1, GST-HDAC2-FLAG, FLAG-HDAC3+FLAG-SMRT and GST-HDAC8) and four class II enzymes (GST-HDAC4-His, His-HDAC6, HDAC7-FLAG and GST-HDAC9-FLAG).

### HDAC assay

Various hydroxamate and non-hydroxamate based HDAC inhibitors (Table 1), were tested against purified rhHDAC proteins using a FDL assay and  $EC_{50}$  values were determined (Table 2). Of the ten inhibitors included in this study, the hydroxamic acid derivatives proved to be the most potent HDAC inhibitors, showing activity on both class I and class II HDAC isoforms. The most potent compounds, TSA, NVP-LAQ824 and panobinostat, exhibited  $EC_{50}$  values in the low nM range against rhHDACs 1, 2, 3, 4 and 9, and were 5-30 fold more potent than vorinostat and belinostat. TSA was also >10 fold more potent than vorinostat and belinostat as an inhibitor of rhHDAC 6 and 7, and was approximately equipotent to belinostat as an inhibitor of rhHDAC 8. NVP-LAQ824 and panobinostat were somewhat more potent as inhibitors of rhHDAC 6 and 7 compared to vorinostat and belinostat, and were equipotent as inhibitors of rhHDAC 8 compared to belinostat. Belinostat was approximately equipotent as an inhibitor of all rhHDAC isoforms tested. Vorinostat exhibited a similar profile, although it appeared to be slightly less effective as an

inhibitor of rhHDAC8. ITF2357 proved to be equipotent to vorinostat and belinostat in the rhHDAC1, 2, 3, 4, 6, and 7 assays.

MS-275 exhibited  $EC_{50}$  values in the nM range against rhHDAC 1. It was significantly less potent ( $EC_{50}$  values in the low  $\mu$ M range) against rhHDACs 2, 3 and 9. MS-275 did not inhibit rhHDACs 4, 6 7 and 8. Similarly, MGCD0103 exhibited  $EC_{50}$  values in the low nM range against rhHDACs 1 and 2. However, it was much less potent against rhHDAC3, with an  $EC_{50}$  value of  $\sim 1 \mu$ M, and did not inhibit the activity of rhHDAC 8 or class II HDACs.

Apicidin inhibited rhHDACs 2 and 3 in the nM range. It also inhibited rhHDAC 8 but with lower potency ( $EC_{50}$  of 575 nM), and had no inhibitory effect on rhHDAC1 and class II rhHDACs. Valproic acid inhibited rhHDACs 1, 2, 3 and 8 in the mM range, but exhibited little activity in the class II rhHDAC assays; hence potency was several orders of magnitude lower compared with the other compounds tested. Those compounds which gave incomplete inhibition of rhHDAC isoforms at 100  $\mu$ M were given a value of  $>100000$ .

In order to make a clear comparison of the potency of the inhibitors on the rhHDAC, log  $EC_{50}$  values were plotted for all compounds with reference to the 2 major HDAC classes (Figure 1a and 1b). Of the class I HDACs, rhHDAC8 was an outlier, as most compounds tested were less potent against this isoform than against rhHDACs 1, 2 and 3 (Figure 1a). MS-275, MGCD0103, apicidin, valproic acid had little activity on class II isoforms, thus confirming that these compounds are class I selective (Figure 1b).

### Cell proliferation assay

Ten hydroxamate and non-hydroxamate HDAC inhibitors (Table 3) were tested in cell proliferation assays in HeLa cells. NVP-LAQ824, panobinostat, TSA and ITF-2357 exhibited the highest potency with  $EC_{50}$ s  $< 1 \mu$ M. MGCD0103, apicidin and belinostat had  $EC_{50}$ s  $< 2 \mu$ M. Vorinostat and MS-275 had an  $EC_{50}$  of 3.2  $\mu$ M and 3.9  $\mu$ M, respectively. VPA had an  $EC_{50}$  of 7.3 mM. Belinostat was found to be 1.7 fold more potent than vorinostat in the HeLa cell proliferation assay.

### Determination of acetylation of levels of proteins after HDACi treatment

The lysates of HeLa cells exposed to HDAC inhibitors at 2 X  $EC_{50}$ , for 4 h and 24 h, were examined by immunoblotting with anti-acetyl-lysine antibody. Both the pan HDAC and the isoform-specific inhibitors induced the acetylation of low molecular weight proteins consistent with the size of histones H3 and H4 (17-kDa and 11-kDa, respectively) in the lysates of cells treated with inhibitor for 24 h. However, the lysates from cells exposed to MS-275 and MGCD0103 for 4 h showed a lower level of acetylation of the two low molecular weight proteins (Figure 2a), compared with lysates from cells treated with the same compounds for 24 h (Figure 2b). In addition, the pan HDAC inhibitors induced the acetylation of a high molecular weight protein, whose size is consistent with that of tubulin (Figure 2a and 2b).

HeLa cells were exposed to the HDACi at 8 X their  $EC_{50}$ , for 24 h, and the whole cell lysates were examined by immunoblotting for acetyl tubulin and acetyl histone H3. The results (Figure 3) show that, compared to the control lysate, the pan HDAC inhibitors (belinostat, vorinostat, TSA, NVP-LAQ824, panobinostat and ITF2357) induced strong acetylation of tubulin and histone H3, while the isoform-specific inhibitors induced strong acetylation of histone H3 but weak or no acetylation of tubulin.

HCC827 and DU145 cells were treated for 24 h with belinostat, MS-275 and MGCD0103 at four different concentrations (0.125 X, 0.25 X, 0.5 X and 1 X  $EC_{50}$ ). The lysates from these cells were examined by immunoblotting with an anti-p53 antibody. The results for both cell lines showed that belinostat decreased the expression of p53 in a dose dependent manner, while the HDAC isoform-specific compounds MS-275 and MGCD0103 did so only weakly, if at all (Figure 4a and b).

### DISCUSSION

Recently published data indicate that both class I and class II HDACs are aberrantly expressed in human cancers. HDAC1 is up-regulated in prostate cancer [24] and

gastric cancer [25], HDAC2 is up-regulated in gastric cancer [26], HDAC3 is up-regulated in lung cancer [27], and there is elevated expression of HDAC6 in oral squamous cell carcinoma [28]. HDAC8 has also recently been implicated in contributing to tumorigenesis by regulating telomerase activity, via its interaction with human ever-shorter telomeres 1B (hEST1B) [29].

The main objective of this study was to compare the potency and specificity of a number of structurally diverse small molecule HDACi compounds as inhibitors of class I or II HDAC isoforms. A number of these inhibitors, including MGCD0103, vorinostat, panobinostat, valproic acid, MS-275, ITF2357 and belinostat, are currently undergoing Phase I, Phase II and Phase III clinical trials as anti-cancer drugs. A further aim was to determine the mode of action of these inhibitors, in particular their effect on the induction of acetylation of specific proteins in cells.

Purified class I and class II HDACs were obtained to address isoform specificity. Four class I and four class II active rhHDAC isoforms were expressed and purified, and although co-purification of a small number of endogenous proteins was noted in the preparations, these were always minor components compared to the recombinant enzyme. The exception was rhHDAC3 preparations, which were all inactive without the presence of SMRT protein. As a result of this requirement, rhHDAC3 was co-expressed with SMRT protein resulting in a highly active enzyme.

Our findings suggest that hydroxamic acid derived compounds such as TSA, NVP-LAQ824, panobinostat, ITF2357, vorinostat and belinostat act as potent pan HDAC isoform inhibitors. A notable observation was the similarity between belinostat and vorinostat in the biochemical isoform assays; both compounds exhibit similar EC<sub>50</sub> values in all but the HDAC8 assay. Although vorinostat and belinostat appear similar in biochemical assays, belinostat is ~ 1.7 - 7 fold more potent as an inhibitor of cell proliferation in a number of cancer cell lines (Table 3; unpublished data from TopoTarget and CuraGen; NCI data (<http://dtp.nci.nih.gov>; Belinostat (NSC# 726630), Vorinostat (NSC# 701852)).

NVP-LAQ824 was more potent than TSA or panobinostat in the HeLa growth-inhibition assay (Table 3). The reason for this is unknown, and is somewhat surprising given the similarity in the enhanced induction of H3/H4 acetylation (Figures 2a, 2b and 3), and the relative equality of these three compounds in biochemical assays of

HDAC inhibition (Table 2). However, it should be pointed out that the biochemical assay is performed in a cell-free system whereas the growth-inhibition assay is performed on intact cells. Potential reasons for the observed enhanced growth-inhibitory activity of NVP-LAQ824, relative to TSA or panobinostat, include: (1) greater cellular uptake or retention of NVP-LAQ824; (2) superior access of NVP-LAQ824 to intracellular targets following uptake; (3) longer half-life of NVP-LAQ824 in intact cells; (4) greater off-target effects of NVP-LAQ824.

In contrast to the hydroxamates, the benzamide compounds MS-275 and MGCD0103, appeared to be HDAC class I specific, as reported previously [30, 31, 32, 33]. Our findings for MS-275 inhibition of HDACs 1, 3 and 8 agree with published data [30].

The small chain fatty acid compound, valproic acid has been shown to inhibit HDACs [34] and to selectively induce proteosomal degradation of HDAC2 [35]. VPA has been used, both as monotherapy and in combination with a number of drugs, in the treatment of cancer [36, 37, 38]. Our data indicates that VPA inhibited class I HDACs 1, 2, 3 and 8 in the millimolar range, but it was ineffective against HDACs 6, 7 and 9.

When comparing the class I HDACs, the EC<sub>50</sub> values for rhHDAC8 were consistently higher for all HDAC inhibitors tested, compared with the other rhHDAC isoforms. This phenomenon may be explained by recent data on HDAC8, which shows that HDAC8 affinity for vorinostat is influenced by the identity of the divalent ion present in the catalytic centre. The K<sub>m</sub> values with Fe(II) and Co(II) were shown to be 5-fold lower than with Zn(II). This data indicates that HDAC8 requires Fe(II) as the divalent ion for catalytic activity, rather than Zn(II) [39].

To determine possible modes of action of the inhibitors, the change in lysine acetylation of key proteins in HeLa cells was established. Inhibition of the HDACs should result in increased acetylation of specific ( $\epsilon$ -N-acetyl lysine) residues on histones. To confirm this hypothesis, HeLa cells were treated with the HDAC inhibitors for 4 h and 24 h; controls were treated with DMSO only. The cell extracts were immunoblotted with anti-acetyl-lysine antibody. Compared with the control extracts, the inhibitor-treated cell extracts showed increased acetylation of two low molecular weight proteins, which corresponded in size to histones H3 and H4. Cells

which had been treated with MS-275 and MGCD0103 for 4 h showed a significantly lower level of acetylation of these proteins compared to pan inhibitor-treated cells. This difference was not apparent after 24 h inhibitor treatment. A possible explanation for this finding is that MS-275 and MGCD0103 may have slower kinetics than the other drugs. These findings indicate that both pan HDAC and class I specific inhibitors bring about similar changes to the level of acetylation of lysine residues on histones H3 and H4.

Interestingly, class II HDACs, particularly HDAC6, appear to be important in a number of key cellular processes. Our data showed that treatment of HeLa cells with HDACi, followed by immunoblotting of the cell extracts with anti-acetylated tubulin antibody resulted in increased tubulin acetylation in extracts treated with the pan HDAC inhibitors; this was not observed with the class I specific inhibitors. There is evidence that HDAC6 destabilizes the microtubule assembly by deacetylating tubulin via its tubulin deacetylase domain [7, 40, 41]. HDAC6 also plays a role in aggresome function [42]. Furthermore, there is evidence that inhibition of HDAC6 results in increased acetylation of HSP90 and disruption of the chaperone association with its client proteins [43]. p53 is a negative regulator of cell growth. It is expressed in the mutant form in a high proportion of tumor cell types. Mutant p53 is known to be a “client” protein of HSP90 [44].

In order to determine if there were any marked differences in p53 levels in cells treated with pan HDAC and class I specific inhibitors, the lysates from DU145 cells and HCC827 cells were immunoblotted with an anti-p53 antibody, following 24 h treatment with HDAC inhibitors. DU145 cells are known to express mutant p53 and HCC827 cells are likely to express p53. Treatment of cells with belinostat showed a dose dependent decrease in p53 levels in the lysates of both cell types. This depletion of mutant p53 was not observed in the lysates from cells treated with the class I inhibitors MS-275 and MGCD0103. These findings collectively emphasize the importance of HDAC6 and suggest that it could also be an important anti-cancer target. If this hypothesis proves to be correct, a pan HDAC inhibitor may be more effective in the clinic than a class I selective inhibitor.

One theoretical consideration is whether inhibition of specific HDACs by selective inhibitors will prove to be less toxic in the clinic than pan-inhibition of both class I

and II HDACs. Evidence available from early phase clinical trials suggests that toxicities associated with pan HDAC inhibitors, such as belinostat and vorinostat [45, 46, 47], are no more severe than those observed in patients treated with class I selective inhibitors, such as MS-275 and MGCD0103 [48, 49, 50]. The most viable conclusion may therefore be, that until further evidence highlighting the benefits of class I selective inhibitors becomes available, pan HDACi may be advantageous since they inhibit important class II HDAC isoforms such as HDAC 6, without demonstrating increased toxicity. However, the ultimate factor(s) deciding between a pan HDAC or class selective inhibitor may depend on the cancer type and/or the chemotherapeutic combination being tested.



## References

1. de Ruijter, A. J., van Gennip, A. H., Caron, H. N, Kemp, S., van Kuilenburg, A. B. (2003) Histone deacetylases (HDACs): characterization of the classical HDAC family. *Biochem J.* **370**, 737-749
2. Cheung, W. L., Briggs, S. D, Allis, C. D. (2000) Acetylation and chromosomal functions. *Curr. Opin. Cell Biol.* **12**, 326-333
3. Cress, W. D., Seto, E. (2000) Histone deacetylases, transcriptional control, and cancer. *J. Cell Physiol.* **184**, 1-16
4. Strahl, B. D., Allis, C. D. (2000) The language of covalent histone modifications. *Nature* **403**, 41-45
5. Wade, P. A. (2001) Transcriptional control at regulatory checkpoints by histone deacetylases: molecular connections between cancer and chromatin. *Hum. Mol. Genet.* **10**, 693-698
6. Thiel, G., Lietz, M., Hohl, M. (2004) How mammalian transcriptional repressors work. *Eur. J. Biochem.* **271**, 2855-2862
7. Matsuyama, A., Shimazu, T., Sumida, Y., Saito, A., Yoshimatsu, Y., Seigneurin-Berny, D., Osada, H., Komatsu, Y., Nishino, N., Khochbin, S., Horinouchi, S., Yoshida, M. (2002) In vivo destabilization of dynamic microtubules by HDAC6-mediated deacetylation. *EMBO J.* **21**, 6820-6831
8. Glaser, K. B., Li J., Pease, L. J., Staver, M. J., Marcotte, P. A., Guo, J., Frey, R. R., Garland, R. B., Heyman, H. R., Wada, C. K., Vasudevan, A., Michaelides, M. R., Davidsen, S. K., Curtin, M. L. (2004) Differential protein acetylation induced by novel histone deacetylase inhibitors. *Biochem. Biophys. Res. Commun.* **325**, 683-690

9. Marks, P., Rifkind, R. A., Richon, V. M., Breslow, R., Miller, T., Kelly, W. K., (2001) Histone deacetylases and cancer: causes and therapies. *Nat. Rev. Cancer.* **1**, 194-202
10. Glozak, M. A., Sengupta, N., Zhang, X., Seto E. (2005) Acetylation and deacetylation of non-histone proteins. *Gene* **363**, 15-23
11. Johnstone, R. W. (2002) Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. *Nat. Rev. Drug Discov.* **1**, 287-299
12. Yoshida, M., Furumai, R., Nishiyama, M., Komatsu, Y., Nishino, N., Horinouchi, S. (2001) Histone deacetylase as a new target for cancer chemotherapy. *Cancer Chemother. Pharmacol.* **48**, Suppl 1 S20-26
13. Yoshida, M., Shimazu, T., Matsuyama, A. (2003) Protein deacetylases: enzymes with functional diversity as novel therapeutic targets. *Prog. Cell Cycle Res.* **5**, 269-278
14. Bertos, N. R., Wang, A. H., Yang, X. J. (2001) Class II histone deacetylases: structure, function, and regulation. *Biochem. Cell Biol.* **79**, 243-252
15. Gregoret, I. V., Lee, Y. M., Goodson, H. V. (2004) Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis, *J. Mol. Biol.* **338**, 17-31
16. Guenther, M. G., Barak, O., Lazar, M. A. (2001) The SMRT and N-CoR corepressors are activating cofactors for histone deacetylase 3. *Mol. Cell Biol.* **21**, 6091-6101
17. Codina, A., Love, J. D., Li, Y., Lazar, M. A., Neuhaus, D., Schwabe, J. W. (2005) Structural insights into the interaction and activation of histone deacetylase3 by nuclear receptor corepressors. *Proc. Natl. Acad. Sci. U S A.* **102**, 6009-6014

18. Fischle, W., Dequiedt, F., Hendzel, M. J., Guenther, M. G., Lazar, M. A., Voelter, W., Verdin, E. (2002) Enzymatic activity associated with class II HDACs is dependent on a multiprotein complex containing HDAC3 and SMRT/N-CoR, *Mol. Cell.* **9**, 45-57
19. Zhang, Y., Gilquin, B., Khochbin, S., Matthias, P. (2006) Two catalytic domains are required for protein deacetylation, *J. Biol. Chem.* **281**, 2401-2404
20. Minucci, S., Pelicci, P. G., Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer, *Nat. Rev. Cancer*, 6(1) (2006) 38-51
21. Yoo C. B., Jones P. A. (2006) Epigenetic therapy of cancer: past, present and future. *Nat. Rev. Drug Discov.* **5**, 37-50
22. McLaughlin, F., La Thangue, N. B. (2004) Histone deacetylase inhibitors open new doors in cancer therapy, *Biochem. Pharmacol.* **68**, 1139-1144
23. Finn, P. W., Bandara, M., Butcher, C., Finn, A., Hollinshead, R., Khan, N., Law, N., Murthy, S., Romero, R., Watkins, C., Andrianov, V., Bokaldere, R. M., Dikovska, K., Gailite, V., Loza, E., Piskunova, I., Stachenkov, I., Vorona, M., Kalvinsh, I. (2005) Novel sulfonamide derivatives as inhibitors of histone deacetylase. *Helvetica Chimica. Acta.* **88** 1630-1643
24. Halkidou K., Gaughan L., Cook S., Leung H. Y., Neal D. E., Robson C. N. (2004) Upregulation and nuclear recruitment of HDAC1 in hormone refractory prostate cancer. *Prostate* **59**, 177-189
25. Choi, J. H., Kwon, H. J., Yoon, B. I., Kim, J. H., Han, S. U., Joo, H. J., Kim, D. Y. (2001) Expression profile of histone deacetylase 1 in gastric cancer tissues. *Jpn. J. Cancer Res.* **92**, 1300-1304

26. Song, J., Noh, J. H., Lee, J. H., Eun, J. W., Ahn, Y. M., Kim, S. Y., Lee, S. H., Park, W. S., Yoo, N. J., Lee, J. Y., Nam, S. W. (2005) Increased expression of histone deacetylase 2 is found in human gastric cancer. *APMIS*. **113**, 264-268
27. Bartling, B., Hofmann, H. S., Boettger, T., Hansen G., Burdach, S., Silber, R. E., Simm, A. (2005) Comparative application of antibody and gene array for expression profiling in human squamous cell lung carcinoma. *Lung Cancer* **49**, 145-54
28. Sakuma, T., Uzawa, K., Onda, T., Shiiba, M., Yokoe, H., Shibahara, T., Tanzawa, H. (2006) Aberrant expression of histone deacetylase 6 in oral squamous cell carcinoma. *Int. J. Oncol.* **29**, 117-124
29. Lee, H., Sengupta, N., Villagra, A., Rezai-Zadeh, N., Seto, E. (2006) Histone deacetylase 8 safeguards the human ever-shorter telomeres 1B (hEST1B) protein from ubiquitin-mediated degradation. *Mol. Cell Biol.* **26**, 5259-5269
30. Hu, E., Dul, E., Sung, C. M., Chen, Z., Kirkpatrick, R., Zhang, G. F., Johanson, K., Liu, R., Lago, A., Hofmann, G., Macarron, R., de los Frailes, M., Perez, P., Krawiec, J., Winkler, J., Jaye, M. (2003) Identification of novel isoform-selective inhibitors within class I histone deacetylases. *J. Pharmacol. Exp. Ther.* **307**, 720-728
31. Eyupoglu, I.Y., Hahnen, E., Trankle, C., Savaskan, N. E., Siebzehnruhl, F. A., Buslei, R., Lemke, D., Wick, W., Fahlbusch, R., Blumcke, I. (2006) Experimental therapy of malignant gliomas using the inhibitor of histone deacetylase MS-275. *Mol. Cancer Ther.* **5**, 1248-1255
32. Kalita, A., Maroun, C., Bonfils, C., Gelmon, K., Siu, L. L., Tolcher, A., Carducci, M., Besterman, J. M., Reid, G. K. and Li, Z. (2005) Pharmacodynamic effect of MGCD0103, an oral isotype-selective histone deacetylase (HDAC) inhibitor, on HDAC enzyme inhibition and histone acetylation induction in phase I clinical trials in patients (pts) with advanced solid tumors or non-Hodgkin's lymphoma (NHL), *J. Clin. Oncol.* **23**, 9631

33. Gelmon, K., Tolcher, A., Carducci, M., Reid, G. K., Li, Z., Kalita, A., Callejas, V., Longstreth, J., Besterman, J. M. and. Siu, L. L., (2005) Phase I trials of the oral histone deacetylase (HDAC) inhibitor MGCD0103 given either daily or 3x weekly for 14 days every 3 weeks in patients (pts) with advanced solid tumors, *J. Clin. Oncol.* **23**, 3147
34. Gurvich, N., Berman, M. G., Wittner, B. S., Gentleman, R. C., Klein, P. S., Green, J. B. (2005) Association of valproate-induced teratogenesis with histone deacetylase inhibition in vivo, *FASEB J.* **19**, 1166-1168
35. Kramer, O.H., Zhu, P., Ostendorff, H. P., Golebiewski, M., Tiefenbach, J., Peters, M. A., Brill, B., Groner, B., Bach, I., Heinzl, T., Gottlicher, M. (2003) The histone deacetylase inhibitor valproic acid selectively induces proteasomal degradation of HDAC2. *EMBO J.* **22**, 3411-3420
36. Kaiser, M., Zavrski, I., Sterz, J., Jakob, C., Fleissner, C., Kloetzl, P. M., Sezer, O., Heider, U. (2006) The effects of the histone deacetylase inhibitor valproic acid on cell cycle, growth suppression and apoptosis in multiple myeloma. *Haematologica.* **91**, 248-251
37. Kuendgen, A., Schmid, M., Schlenk, R., Knipp, S., Hildebrandt, B., Steidl, C., Germing, U., Haas, R., Dohner, H., Gattermann, N. (2006) The histone deacetylase (HDAC) inhibitor valproic acid as monotherapy or in combination with all-trans retinoic acid in patients with acute myeloid leukemia. *Cancer* **106**, 112-119
38. Morotti, A., Cilloni, D., Messa, F., Arruga, F., Defilippi, I., Carturan, S., Catalano, R., Rosso, V., Chiarenza, A., Pilatino, C., Guerrasio, A., Taulli, R., Bracco, E., Pautasso, M., Baraban, D., Gottardi, E., Saglio, G. (2006) Valproate enhances imatinib-induced growth arrest and apoptosis in chronic myeloid leukemia cells. *Cancer* **106**, 1188-1196

39. Gantt, S. L., Gattis, S. G., Fierke, C. A. (2006) Catalytic activity and inhibition of human histone deacetylase 8 is dependent on the identity of the active site metal ion. *Biochemistry* **45**, 6170-6178
40. Hubbert, C., Guardiola, A., Shao, R., Kawaguchi, Y., Ito, A., Nixon, A., Yoshida, M., Wang, X. F., Yao, T. P. (2002) HDAC6 is a microtubule-associated deacetylase. *Nature* **417**, 455-458
41. Zhang, Y., Li, N., Caron, C., Matthias, G., Hess, D., Khochbin, S., Matthias, P. (2003) HDAC-6 interacts with and deacetylates tubulin and microtubules in vivo. *EMBO J.* **22**, 1168-1179
42. Kawaguchi, Y., Kovacs, J. J., McLaurin, A., Vance, J. M., Ito, A., Yao, T. P. (2003). The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. *Cell.* **115**, 727-738
43. Bali, P., Pranpat, M., Bradner, J., Balasis, M., Fiskus, W., Guo, F., Rocha, K., Kumaraswamy, S., Boyapalle, S., Atadja, P., Seto, E., Bhalla, K. (2005) Inhibition of histone deacetylase 6 acetylates and disrupts the chaperone function of heat shock protein 90: a novel basis for antileukemia activity of histone deacetylase inhibitors. *J. Biol. Chem.* **280**, 26729-26734
44. Sharp, S., Workman, P. (2006) Inhibitors of the HSP90 molecular chaperone: current status. *Adv. Cancer Res.* **95**, 323-348
45. Steele, N., Vidal, L., Plumb, J., Attard, G., Rasmussen, A., Buhl-Jensen, P., Brown, R., Blagden, S., Evans, J., de Bono, J., A phase 1 pharmacokinetic (PK) and pharmacodynamic (PD) study of the histone deacetylase (HDAC) inhibitor PXD101 in patients (pts) with advanced solid tumours, *J. Clin. Oncol.* (Meeting Abstracts) 23 (2005) 3035
46. Duvic, M., Vu, J. (2007) Vorinostat: a new oral histone deacetylase inhibitor approved for cutaneous T-cell lymphoma. *Expert Opin Investig Drugs* **16**, 1111-1120

47. Olsen, E. A., Kim, Y. H., Kuzel, T. M., Pacheco, T. R., Foss, F. M., Parker, S., Frankel, S. R., Chen, C., Ricker, J. L., Arduino, J. M., Duvic, M. (2007) Phase IIb multicenter trial of vorinostat in patients with persistent, progressive, or treatment refractory cutaneous T-cell lymphoma. *J. Clin. Oncol.* **25** 3109-3115
48. Rasheed, W. K., Johnstone, R. W., Prince, R. M. (2007) Histone deacetylase inhibitors in cancer therapy. *Expert Opin Investig Drugs* **16**, 659-678
49. Piekarz, R. L., Sackett, D. L., Bates, S. E. (2007) Histone deacetylase inhibitors and demethylating agents: clinical development of histone deacetylase inhibitors for cancer therapy. *Cancer J.* **13**, 30-39
50. Marchion, D., Munster, P. (2007) Development of histone deacetylase inhibitors for cancer treatment. *Expert Rev Anticancer Ther.* **74**, 583-598

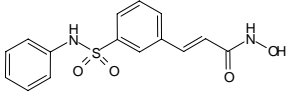
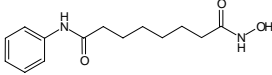
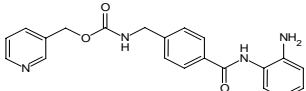
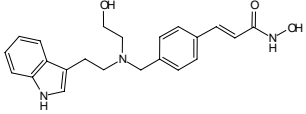
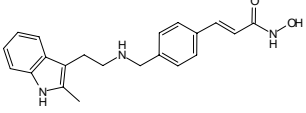
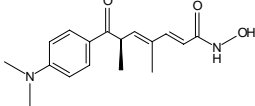
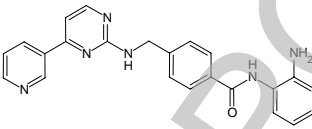
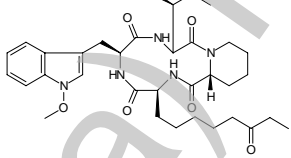
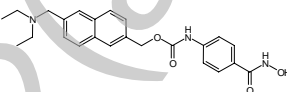
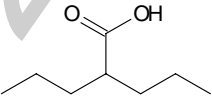
Compound	Structure	Chemical Class	Development status
Belinostat		Hydroxamate	Phase II
Vorinostat		Hydroxamate	Launched
MS-275		Benzamide	Phase II
NVP-LAQ824		Hydroxamate	Phase I
Panobinostat		Hydroxamate	Phase II
TSA		Hydroxamate	Preclinical
MGCD0103		Benzamide	Phase II
Apicidin		Cyclic tetrapeptide	Preclinical
ITF2357		Hydroxamate	Phase I
Valproic Acid		Short chain fatty acid	Phase II

Table 1. HDAC inhibitors



Inhibitor	EC <sub>50</sub> ± SEM (nM)							
	rhHDAC1	rhHDAC2	rhHDAC3	rhHDAC4	rhHDAC6	rhHDAC7	rhHDAC8	rhHDAC9
<b>TSA</b>	2 ± 0	3 ± 0	4 ± 1	6 ± 2	3 ± 1	5 ± 2	456 ± 59	6 ± 5
<b>NVP-LAQ824</b>	5 ± 1	5 ± 1	12 ± 1	23 ± 18	7 ± 3	18 ± 4	162 ± 44	6 ± 5
<b>Panobinostat</b>	3 ± 0	3 ± 0	4 ± 1	12 ± 5	61 ± 1	14 ± 7	248 ± 11	3 ± 2
<b>ITF2357</b>	28 ± 8	56 ± 13	21 ± 3	52 ± 5	27 ± 16	163 ± 8	ND	ND
<b>Belinostat</b>	41 ± 6	125 ± 21	30 ± 0	115 ± 16	82 ± 19	67 ± 22	216 ± 43	128 ± 46
<b>Vorinostat</b>	68 ± 14	164 ± 45	48 ± 17	101 ± 31	90 ± 26	104 ± 35	1524 ± 463	107 ± 21
<b>MS-275</b>	181 ± 62	1155 ± 134	2311 ± 803	>10,000	>10,000	>10,000	>10,000	505 ± 37
<b>MGCD0103</b>	34 ± 17	34 ± 8	998 ± 431	>10,000	>10,000	>10,000	>10,000	ND
<b>Apicidin</b>	>10,000	120 ± 28	43 ± 7	>10,000	>10,000	>10,000	575 ± 111	>10,000
<b>Valproic acid</b>	1,584,000 ± 302,642	3,068,000 ± 0	3,071,000 ± 0	ND	>10,000	>10,000	7,442,000 ± 2,740,000	>10,000

Table 2. Inhibition of rhHDAC isoforms in the HDAC *in vitro* biochemical assay. Each compound was assayed in triplicate per plate. EC<sub>50</sub> values were determined from the average of a minimum of 2 plates. The average EC<sub>50</sub> values and standard error from the mean are shown in nanomolar units

Inhibitor	EC <sub>50</sub> ±SEM (μM)
TSA	0.187 ± 0.095
NVP-LAQ824	<0.04
Panobinostat	0.106 ± 0.040
ITF2357	0.408 ± 0.183
Belinostat	1.921 ± 0.289
Vorinostat	3.245 ± 1.175
MS-275	3.919 ± 1.370
MGCD0103	1.030 ± 0.294
Apicidin	1.550 ± 0.919
Valproic acid	7,275 ± 247

Table 3. Inhibition of HeLa cell proliferation by HDAC inhibitors. Each compound was assayed in triplicate per plate. EC<sub>50</sub> values were determined from the average of a minimum of 2 plates. The average EC<sub>50</sub> values and standard error from the mean are shown in micromolar units

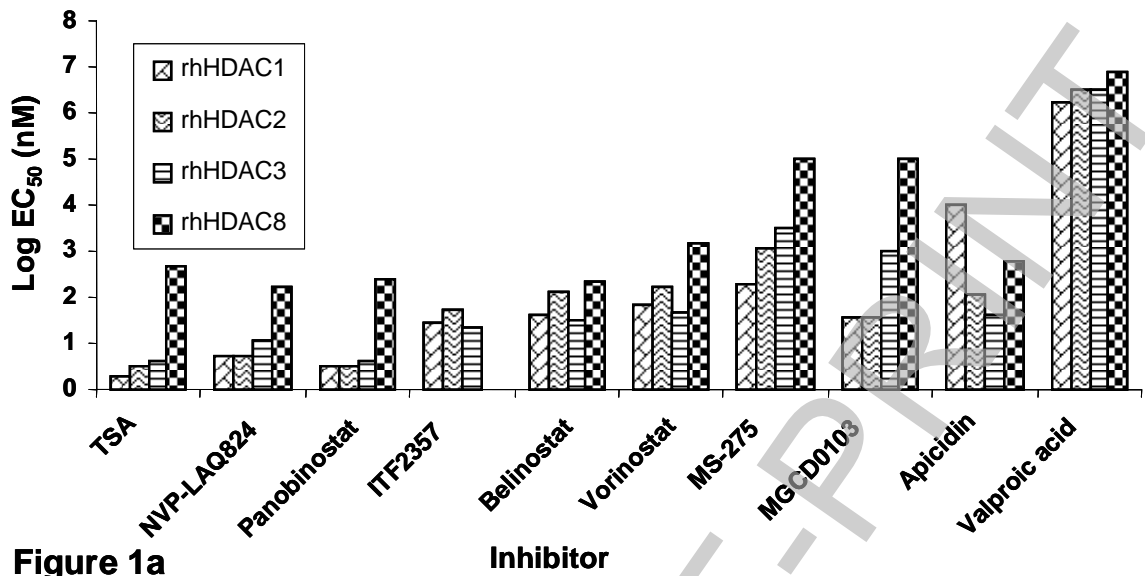
## Figure Legends

Figure 1. The inhibitory activity of the indicated HDACi on recombinant class I [1, 2, 3, 8; (a)] and class II [4, 6, 7, 9; (b)] HDAC isoforms was determined in a cell-free system using the Fluor de Lys™ assay and plotted as Log EC<sub>50</sub> for purposes of comparison

Figure 2. HeLa cells were exposed to the indicated HDACi at a concentration of 2 X EC<sub>50</sub> (as determined in a cell viability assay on HeLa cells) for 4 h and 24 h, after which time total cell lysates were prepared and examined by immunoblotting under reducing conditions with an antibody to acetyl-lysine. The higher molecular weight protein (~50 kDa) is likely alpha-tubulin and the lower molecular weight proteins (~10-15 kDa) are likely histones. The blots were reprobbed with actin to confirm equal loading

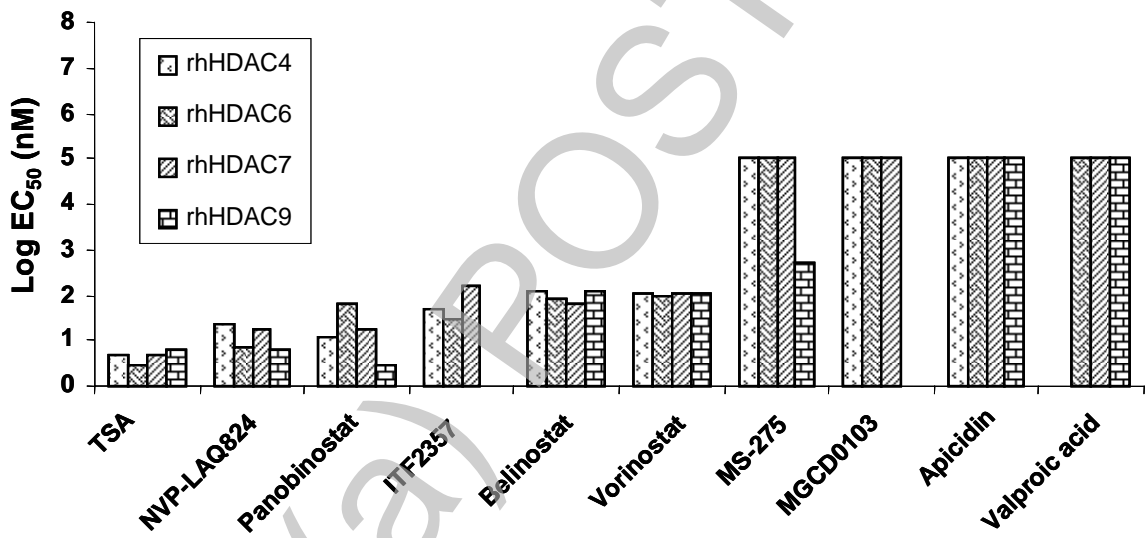
Figure 3. HeLa cells were exposed to the indicated HDACi at a concentration of 8 X EC<sub>50</sub> (as determined in a cell viability assay on HeLa cells) for 24 h, after which time total cell lysates were prepared and examined by immunoblotting under reducing conditions with antibodies to acetyl alpha-tubulin or acetyl-histone H3. The blots were reprobbed with actin to confirm equal loading

Figure 4. HCC827 and DU145 cells were exposed to the indicated HDACi at concentrations of 0.125-1 X EC<sub>50</sub> (as determined in a cell viability assay on HeLa cells) for 24 h, after which time total cell lysates were prepared and examined by immunoblotting under reducing conditions with an antibody to p53. The blots were reprobbed with actin to confirm equal loading



**Figure 1a**

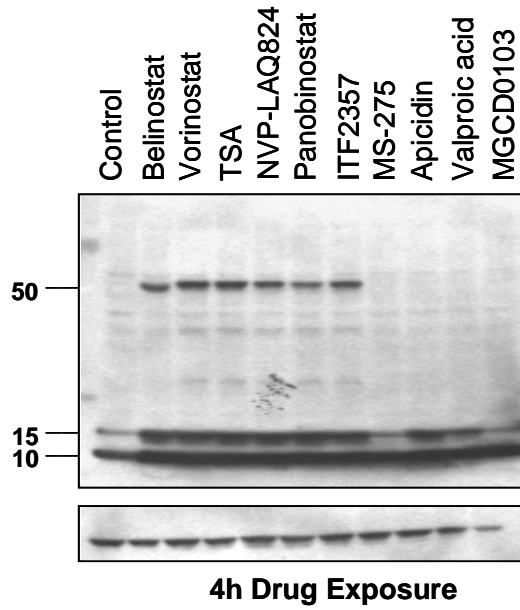
**Inhibitor**



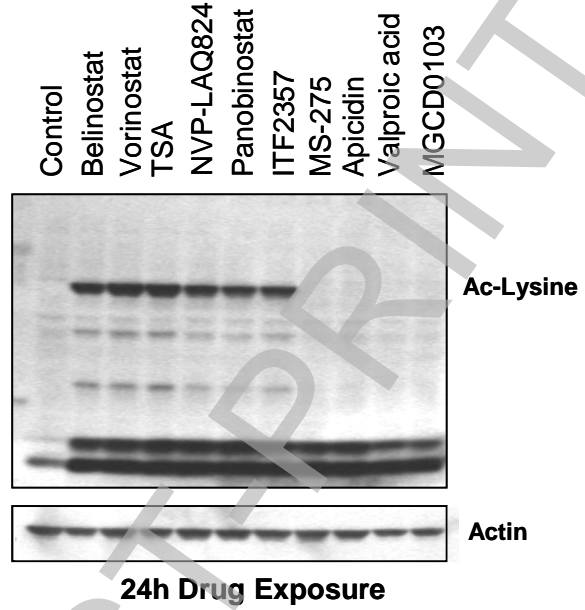
**Figure 1b**

**Inhibitor**

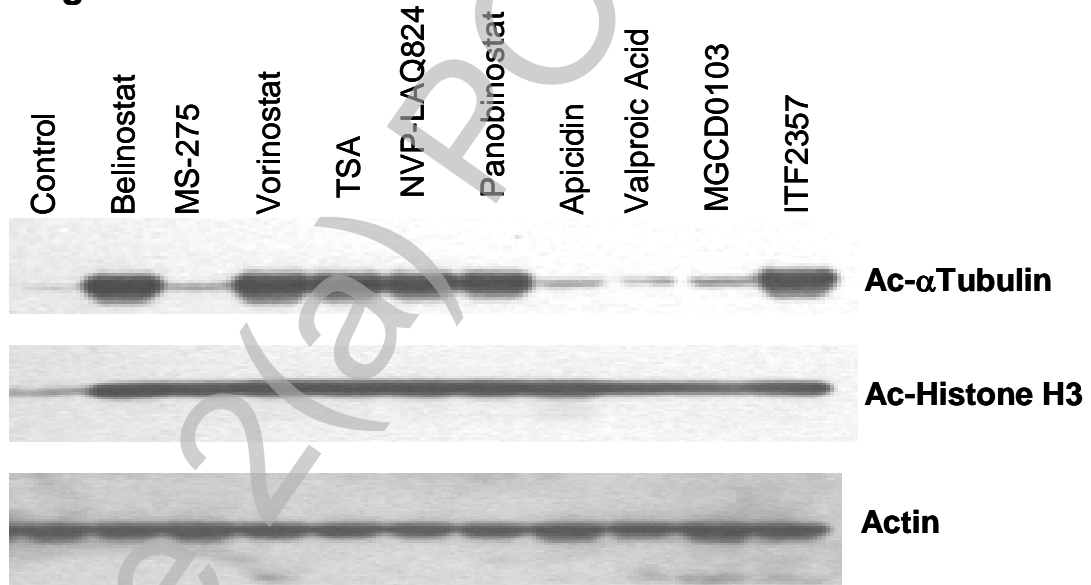
**Figure 2a**



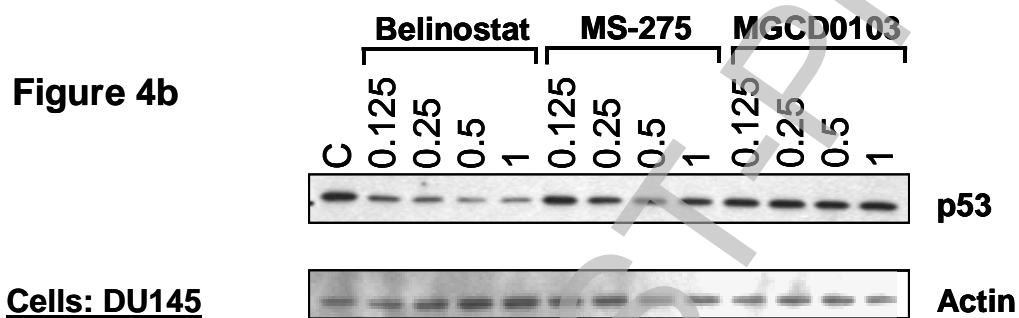
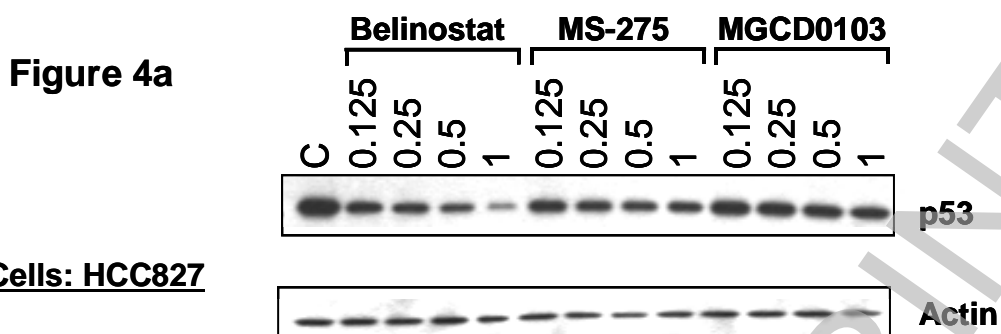
**Figure 2b**



**Figure 3**



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