Histone Deacetylase Cytoplasmic Trapping by a Novel Fluorescent HDAC Inhibitor

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Abbreviations List: HDACIs (histone deacetylase inhibitors), SAHA (suberoylanilide hydroxamic acid), DMSO (dimethylsulfoxide), IC50 (inhibitory concentrations 50%), GI50 (50% cell growth inhibition)

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Abstract

Inhibitors of histone deacetylases (HDACs) are an important emerging class of drugs for the treatment of cancers. HDAC inhibitors are currently under evaluation in clinical trials as single agents and as sensitizers in combinations with chemotherapies and radiation therapy. Although these drugs have important effects on cancer cell growth and functions, the mechanisms underlying HDAC inhibitor activities remain to be fully defined. Using rational drug design, compound 2, a fluorescent Class II HDAC targeting inhibitor, was synthesized, and observed to accumulate in the cytoplasmic compartments of treated cells, but not in the nuclei. Furthermore, immunostaining of inhibitor exposed cells for HDAC4 demonstrated accumulation of this enzyme in the cytoplasmic compartment with concomitant increased acetylation of tubulin and nuclear histones. These observations support a mechanism by which nuclear histone acetylation is increased as a result of HDAC4 trapping and sequestration in the cytoplasm after binding to compound 2. The HDAC inhibitor offers potential as a novel theranostic agent, combining diagnostic and therapeutic properties in the same molecule.
Introduction

The current understanding of the roles of acetylation in the epigenetic regulation of chromatin structure, and gene expression rests on the balance of activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs). Increased acetylation of histones leads to changes in chromatin structure and accessibility for key cellular proteins to specific target sites (1, 2). HATs acetylate lysine groups at the amino terminal tails of nuclear histones to neutralize positive charges on the histones, yielding a more open, transcriptionally active chromatin structure (3). In contrast, the HDACs deacetylate and suppress transcription. In this model, inhibitors of HDACs bias the balance toward a more acetylated state. Such a shift in the relative activities of these enzymes may change gene expression necessary for DNA repair, replication, cell cycle checkpoint activation and tumor suppression (4, 5).

Human histone deacetylases can be divided into four classes based on structure, sequence homology, and domain organization. Class I consists of HDACs 1, 2, 3, and 8. Class I HDACs are nuclear and play roles in cell proliferation and apoptosis. Class II includes HDACs 4, 5, 6, 7, 9, and 10 (6). These enzymes are characterized by a large NH₂-terminal domain or a second catalytic site and their expression is more restricted, suggesting roles in cellular differentiation and development (2). Class III enzymes include the SIRTs (sirtuins), and are NAD-dependent deacetylases (7). Class III enzymes are not inhibited by TSA or other hydroxamates. HDAC11 is designated as Class IV based on phylogenetic analysis (8).

HDACs are found in the nuclear and cytoplasmic compartments. Although they are involved in critical cellular functions, such as cell cycle regulation and apoptosis, a key function of HDACs is transcriptional regulation. HDACs function as components of large multi-protein complexes that bind to promoters and repress transcription. While Class I HDACs are predominantly sub-localized in the nucleus, Class II HDACs shuttle between the nucleus and the cytoplasm; however, both classes of HDACs have conserved deacetylase core domains of
approximately 400 amino acids and zinc binding sites. It is the core domain that presents the principal target for design of inhibitory small molecules. Key reported acetylation mechanisms relevant to cancer treatment have involved histones and tubulin, as well as a variety of other non-histone proteins (9-12). We have reported radiation sensitizing properties of novel hydroxamic acid and mercaptoacetamide inhibitors in a search for HDAC isomer specificity (13, 14). By designing and synthesizing new class II targeting HDAC inhibitors offering intrinsic fluorescent properties, we provide further insight into action mechanisms and sub-cellular sites of inhibitor activities.

**Materials and Methods:**

**Materials**

HeLa nuclear extracts and fluorimetric histone deacetylase assay kits were obtained from BIOMOL (Plymouth Meeting, PA). Antibodies for acetylated α-tubulin and HDAC4 were purchased from Sigma, acetylated histone H4 from Upstate Biotechnology (Lake Placid, NY), and GAPDH from Trevigen (Gaithersburg, MD).

As a quality control and in order to confirm the identity of the cultured cell lines used in these experiments, cell lines were submitted to genetic fingerprinting. The commercially available Cell IDTM System (Promega) was used to screen cell lines obtained from the Georgetown Tissue Culture Shared Resource (LNCaP). Cells which were obtained from the ATCC (A549, MCF7, C42, PC-3) were genetically fingerprinted using the PowerPlex 1.2 System by the ATCC and have been passaged for less than six months before use in these experiments. All cell lines were screened for the presence of mycoplasms prior to use by the
Georgetown Tissue Culture Shared Resource using the Gen-Probe Mycoplasma Tissue Culture NI Rapid Detection System.

Synthesis

Schemes, methods of syntheses and spectroscopic data for the new compounds are presented in the supplementary material. Purity and identity were established for all compounds using mass spectrometry, NMR spectrometry and HPLC analyses.

HDAC Assays

The values of IC_{50} concentrations of HDAC inhibitors were determined performing a fluorimetric histone deacetylase assay following the manufacturer’s instructions. For the pan-HDAC assay, HeLa nuclear extracts were used as a source of histone deacetylase, and the histone deacetylase isomer inhibition assays were performed using purified recombinant histone deacetylase proteins of the various isomers (BPS Bioscience Inc, CA). Reactions were prepared in 0.1 M KCl, 20 mM Hepes/NaOH at pH 7.9, 20% glycerol, 0.2 mM DTA, 0.5 mM DTT, and 0.5 mM PMSF. The HDAC assay was performed using Fluor de Lys substrate and concentrations (nM to µM) of compounds at 37 °C in HDAC assay buffer, containing 25 mM Tris/Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, and 1 mM MgCl_2. Fluorescence was measured with an excitation at a wave-length of 360 nm and emitted light of 460 nm (TECAN ULTRA 384) was detected. Negative (no enzyme, no inhibitor, a drug with no HDAC inhibition activity) and positive controls (a HeLa nuclear extract with no HDAC inhibitor and known HDAC inhibitor SAHA) were included in the assay reactions. The reaction was performed in triplicate for each sample. Each point represents the mean ± SD of replicates.

Cell Growth Inhibition Assay
Cell lines PC-3 (prostate cancer), C42 (androgen-independent prostate cancer), and LNCap (androgen-dependent prostate cancer) were cultured in RPMI-1640 medium supplemented with 100 mg/mL of streptomycin, 100 mg/mL of penicillin, 2 mM L-glutamine, 1 mg/mL of hydrocodisone, and 20% fetal bovine serum. These cultures were maintained in a 95% air/5% CO₂ incubator at 37 °C. After 24 hours seeding, a solution of the compound or DMSO vehicle control were added to cells and incubated for 48 hours at 37 °C. Growth inhibition was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay according to manufacturer's instructions (Promega, Inc. Madison, WI). The absorbance was measured at 490 nm on a microplate reader. The 50% growth inhibition (GI₅₀) was calculated as the compound concentration required reducing cell number by 50% compared with control.

**Cell Cycle Distribution**

LNCap and PC-3 cells were incubated in the presence or absence of compound prior to harvesting at the indicated time points, washed twice with cold phosphate-buffered saline, and 1-2 × 10⁶ cells were resuspended in 0.5 mL of ice-cold phosphate-buffered saline for analysis. Single cell suspensions were verified microscopically. The cell suspension was gradually mixed with ice-cold absolute ethanol to the final volume of 2.0 mL, and cooled on ice at least for 20 min before staining with propidium iodide. The cell cycle distributions were measured in a fluorescence-activated cell sorter from Becton Dickinson. DNA content levels were quantified using ModFit LT 3.0 software (Verity Software House, Inc.).

**Fluorescent Measurement**

Cells were plated onto glass slides at a density of 500,000 per slide and incubated overnight. Cells were replenished with a medium containing 20 μM of compound 2 and incubated for one hour. Slides were washed with phosphate-buffered saline, cells were fixed
with a 4% solution of formaldehyde for ten minutes, washed and mounted. Nuclear staining was achieved by treating cells with propidium iodide (1:1000) for 4 minutes after fixation. The fluorescent compound was imaged using a multiphoton laser with an excitation wavelength of 700 nm and an emission wavelength of 510 nm. Propidium iodide was detected with an excitation wavelength of 535 nm and an emission wavelength of 617 nm. Images were taken at 63X.

**HDAC4 Antibody and Confocal Detection**

PC3 cells were treated with drug at the final concentration of 1 µM for 24 hours. The cells were then washed with 1 × PBS and fixed with 4% percent paraformaldehyde for 15 min. Non-specific binding was blocked with 10% normal goat serum in PBS containing 0.2% tween-20 (Sigma Aldrich P7949) for one hour. Cells were treated with mouse monoclonal anti-HDAC4 antibody (Sigma Aldrich H0163) 1:200 dilution in 5% goat serum in PBS for one hour at room temperature. Fluorescent rabbit anti-mouse monoclonal antibodies (Alexa fluor 488 from Invitrogen A11001) were applied to the cells for 30 minutes. After washing, Vecta shield mounting medium for fluorescence with DIPA (Vector laboratory H-1200) were loaded on the cells and covered with cover glass for imaging.

**Western Blot Analysis**

Cells were treated 24 hours after seeding with compound (dissolved in DMSO) at the concentration of (0.1, 1, 5, 10 and 50 µM), equal amount of DMSO was added to the control groups. After incubation at 37 °C, the cell culture media was removed and washed once with fresh media, then, HDAC compounds were added to the cell culture. The cell culture was kept at 37 °C for another 24 hours, then harvested. The cells were harvested by washing two times
with PBS and lysed with RIPA buffer. The protein concentrations were measured by Bradford protein assay method. Equal amounts of proteins were mounted on the 4-20% SDS-PAGE gel, with antibody against acetylated $\alpha$-tubulin (Sigma) and acetylated histone H3 (Cell Signaling). For Western Blot analysis, proteins were separated in 4-20% SDS-PAGE gels and blotted onto ImmobilonP membrane (Millipore, Bedford, MA). ECL detection (Amersham, Piscataway, NJ) was performed according to manufacturer’s instructions.

**Results**

**Rational Drug Design and Molecular Modeling**

We developed homology models to represent HDAC isomers based on the observation that each HDAC is unique in the CAP region. Substitution of bulky cap groups potentially offers increased selectivity and potency, and the site is capable of accommodating a large fluorescent probe. Docking simulations of compounds 1-4 (Figure 1A) against HDAC isomers led to comparisons of root mean standard deviations (RMSD) and molecular dynamics (MD) simulations on HDACs 6, 7 and 8. The results of 100ps molecular dynamic simulations of compound 2 are illustrated in Figures 1B-D. The planar ring of the dansyl moiety contributes to affinity by preferentially occupying the relatively planar and hydrophobic portion of the CAP region (Figure 1B-D).

**Synthesis of Compound 2 and Analogs**

The strategy for synthesis of fluorescent molecule 2 and analogs containing various length linkers is outlined in Supplemental Figure 1. In general, dansyl chloride was reacted with the appropriate amino carboxylic acid to yield the dansylated derivative acid 5-8 in high yield. Using an EDCI-HOBT combined condensation, acids 5-8 were activated and condensed with benzyloxyamine to yield amides 9-12. Hydrogenation of 9-12 provided the final hydroxamic acid products 1-4 with yields over 90%.
Screening for HDAC Inhibitory Activity

In vitro pan-HDAC activity inhibition assays were performed as the initial screen to assess overall compound potencies. Compound 2 and 3 are effective pan-HDAC inhibitors with IC₅₀ values of 125 nM and 116 nM, respectively as compared to compounds 1 and 4 (Table 1). The inhibitory activities of these compounds were linker-length dependent. The four and seven carbon spacers (compound 1 and 4) were less active than were the five and six carbon linkers (compound 2 and 3), which allowed appropriate positioning of the hydroxyamic acid and the dansyl fluorophore within the hydrophobic cavity (Figures 1B-D).

HDAC inhibitory potency of compound 2 was also maintained by substituting a sulfonamide in the linker unit in place of a carbonylamide. The dansyl moiety is exposed in the CAP region and forms vertical pi-pi stacking interactions with surrounding hydrophobic residues. Compound 3 contains a six carbon spacer flexibly positioned into the hydrophobic cavity with hydrogen bonding similar to that of compound 2 (Structure figure not shown), however, the seven carbon linker in compound 4 does not position the dansyl group optimally (figure not shown). Although the four carbon spacer of compound 1 fits well into the hydrophobic site, the lack of H-bond formation of the amide group (contained in the dansyl moiety) with the surface residues results in low affinity (Table 1). These data demonstrate that compounds containing dansyl groups in the cap regions offer effective inhibition of HDACs.

The compounds fall well within the rules proposed by Lipinski (15) and we also note that the polar surface areas of the compounds are all greater than 150 Å². Measurements of the excitation and emission spectra for compounds 1-4 document the fluorescence properties of the HDAC inhibitors (Table 1).

HDAC Isomer Specificity
Observations of non-overlapping functions for HDAC isomers present an opportunity for achieving therapeutic specificities. Compounds 2 and 3 were evaluated for inhibitory effects on recombinant proteins available for ten of the class I and II HDAC isomers. As summarized in Table 2, compounds 2 and 3 show increased inhibition of class II HDAC isomers. Of note, HDAC6 is a microtubule associated protein that can deacetylate α-tubulin in vitro. Also, HDAC6 has been reported to interact with β-tubulin in vitro (16). This dual deacetylase function of HDAC6 may offer a research tool to study cellular function of HDACs. Because HDAC6 localizes predominantly in the cytoplasm and deacetylates tubulin, we considered it as functionally distinct from other known HDACs, and focused on substrates other than histones.

Figure 1 shows the molecular model of compound 2 in complex with HDAC6 (panel 1B), HDAC 7 (panel 1C), and HDAC8 (panel 1D). Interactions of the dansyl moiety within the CAP regions of HDAC isomers provide a potential basis for selectivity. Comparison of HDAC isomers 6 and 8 reveals that the shape of the HDAC6 binding cavity differs significantly. This data along with differences in HDAC isomer inhibition further support that there may be significant conformational or structural dissimilarity in class I and class II HDAC isomers. Furthermore, electrostatic and steric surface interactions around the ligand-binding channel show differences between class I and class II HDACs.

Cell Growth Inhibition and Cell Cycle Distributions

As promising targets for cancer treatment, HDAC inhibitors have shown significant anti-proliferative and apoptotic activities on various cancer cells, including prostate cancer (17-19). HDAC inhibitors are capable of inducing differentiation and/or apoptosis of prostate cancer cells. Therefore, we evaluated compound 2 for growth inhibition of three human prostate cancer cell lines including PC-3, C4-2 and LNCap. Compound 2 inhibited the growth of all three prostate cancer cell lines with GI50’s for PC3 (1.54 ± 0.27 μM), C4-2 (1.91 ± 0.15 μM) and LNCaP (1.30 ±
0.41 μM). Treatment with GI_{50} doses of compound 2 resulted in an increase of the percentage of cells in G₁ as compared to S or G₂ (Table 3). This effect was observed in both androgen dependent and independent prostate cancer cells. We also evaluated compound 2 against additional human cancer cell lines MCF7 and A549 (Table 3). The effects of our compound on cell cycle distributions were similar to that observed with SAHA.

### Imaging Compound 2 in Human Prostate Cancer Cells

Utilizing the inherent fluorescent properties of our compound, we imaged compound 2 in human prostate cancer cells with confocal microscopy. The addition of 20 μM of compound 2 to PC3 (Supplemental figure 2A-C) and DU145 (Supplemental figure 2D-F) cells demonstrated intracellular cytoplasmic delivery. The images shown here were obtained 60 minutes after cell treatment. Similar results were obtained after 8 hrs and 16 hrs, confirming that compound 2 localized to the cytoplasm. Co-localization experiments with propidium iodide reveal that compound 2 does not localize to nuclei (Figure 2A).

### Imaging the Effects of Compound 2 on Histone Acetylation

Next, we determined the effects of compound 2 on histone acetylation in A549 cells. A549 cells were treated with 20 μM of compound 2, allowing comparisons to previously published data (20) and evaluation of this novel compound in other cancer cell types. Figure 2B demonstrates that compound 2 increases acetylation of nuclear histones, a marker of the functional effect on the enzyme as compared to controls which was not shown clearly in the literature (Figure 2B(f)) (20). Interestingly, compound 2 localized to the cytoplasm (Figure 2B(d)) and not in the nucleus, prompting a reevaluation of the accepted mechanisms of HDAC inhibitor action in the nucleus (21, 22).
Imaging the Effects of Compound 2 on Non-Histone Protein

Class II HDACs shuttle between the nuclei and cytoplasmic compartments of cells, and modify the acetylation status of tubulin residing in the cytoplasm. Compound 2 also caused an increase in $\alpha$-tubulin acetylation (Figure 2C) in the cytoplasms of A549 cells as compared to control (Figure 2C(e)). As shown in experiments using other cell lines, compound 2 fluoresced in the cytoplasms of these cells as well (Figure 2C(b)).

Mechanistic Studies on HDAC4 Interactions with Compound 2

HDAC isomers demonstrate non-redundant functions. HDAC6 is a microtubule associated protein that deacetylates tubulin in vitro. The expression of HDAC6 has been reported exclusively in the cytoplasm (23). HDAC10 was found to be the closest relative of HDAC6, but it has an incomplete second HDAC domain (24-26). Other class II HDACs, for example HDAC4 and HDAC5, have been found to shuttle between the nucleus and the cytoplasm (27, 28), whereas HDAC7 shuttling is not certain. This correlates to the observations that compound 2 is potent in inhibiting HDAC 4, 5, 6 and 10, and also has shown activity against HDAC11, which combines properties of both class I and class II HDACs (Table 2). We interpret these data to show that compound 2 affects the functions of the nuclear HDAC enzymes without entering the nucleus.

Compound 2 fluorescence was detected in the cytoplasm; therefore, we sought to evaluate its interactions with HDAC’s capable of shuttling from the nucleus into the cytoplasm. HDAC4 was selected as the model HDAC for these studies given its known cytoplasmic-nuclear shuttling (29) and the potency (IC$_{50}$, Table 2) of compound 2 against HDAC4. Using an antibody to HDAC4, we evaluated HDAC4 distribution in PC3 cells with/without inhibitor treatment (Figure 3A-C). Confocal microscopy shows the expected cytoplasmic and nuclear distributions of HDAC4 (Figure 3A). Treatment of PC3 cells with 1 $\mu$M of compound 2 resulted
in a significant reduction of HDAC4 in the nucleus, DAPI staining of the nucleus confirmed depletion of HDAC4 (Figure 3B). Interestingly, in control experiments, SAHA also depleted the nucleus of HDAC4 (Figure 3C). As a control, we evaluated by western analysis of five HDAC isomers including HDAC1 and 2 and found no change in protein concentration up to 50 μM dose (See supplemental Figure 3). In addition, PC3 cells treated with compound 2 and immunostained for HDAC1, continued to show HDAC1 in nucleus (See supplemental Figure 4). These observations demonstrate the utility of the new HDAC ligand and point to potential new interpretations of mechanisms for inhibition of nuclear HDAC-mediated deacetylation.

The transcription of p21 is regulated through acetylation of histone. Acetylation of histones results in increased p21 transcription. Hence, the expression levels of p21 reflect the inhibitory effects on HDAC’s by these compounds. PC3 cells do not express p53, resulting in decreased p21 levels (30, 31). T24 cells (human bladder cancer cells) were used to check the expression levels of p21 following treatment with compound 2. Exposure of T24 cells to 5 μM of compound 2 resulted in up-regulated expression of p21 (Figure 4A).

p21 binds to and inhibits cyclin-CDK2 resulting in G1 arrest (32). Compound 2 caused G1 arrest in PC3 and LNCaP cells (Table 3). Since PC3 cells do not express p53, these data suggest the presence of an alternative pathway to G1 arrest.

HDACs play important roles in regulating other cellular activities. Class II HDAC shuttle between the nuclei and cytoplasm, and modify the acetylation status of tubulin residing in the cytoplasm. When PC3 cells were treated with compound 2 or with SAHA, the acetylation levels of tubulin (Figure 4B) and H3 (Figure 4C) were increased. This further supports our findings with the HDAC isomer inhibition assay (Table 2). Compound 2 showed potent inhibition of class II HDACs, especially HDAC6.
HDAC6 is known to affect protein stability (33), a potentially increasing degradation of class I HDACs. We evaluated five HDAC isomers including HDAC1, 2, 3, 4 and 5 and observed no changes in protein levels up to 50 μM exposure (Supplemental figure 3).

Discussion

In this study we have designed and synthesized novel fluorescent HDAC inhibitors. We report compounds 2 and 3, HDAC class II selective hydroxamate inhibitors, especially targeting HDAC4 and HDAC6. Compound 2 not only exhibited general properties of hydroxamate HDAC inhibitors, inhibiting cancer cell growth and causing G1 arrest, but was also found exclusively in the cytoplasm of cells. Interestingly, treatment of cells with compound 2 resulted in increased nuclear acetylation. We attribute this observation to HDAC4 sequestration, shifting the balance of histone acetylation to a more acetylated state. This compound also inhibited tubulin deacetylation in the cytoplasm of human lung cancer cells, supporting its action on HDAC6. These activities are promising for developing the compounds as potential cancer therapies.

Our results demonstrating the localization of a hydroxamate HDAC inhibitor in the cytoplasm may offer a new paradigm for understanding how HDAC inhibitors affect nuclear function of HDACs. This mechanism may be related to “HDAC trapping” of shuttled proteins. Cytoplasmic localization of compound 2 further leads to speculation on 1) the effects of compound 2 on nuclear translocation of HDAC proteins, 2) potential inhibitory effects on the process of mRNA nuclear exportation, 3) cytoplasmic HDAC degradation, and 4) effects of compound 2 on protein folding or interactions with nuclear translocation or chaperone proteins. Also, restriction of HDAC4 in the cytoplasm may result in disruption of nuclear complexes that include HDAC4.

Taken together, these observations suggest that compound 2 leads to modified
expression and functions of molecules important for cellular responses by confining HDACs to the cytoplasmic compartments. This finding provides a valuable tool for further studies to understand the mechanism of the action of HDAC inhibitors. Further studies of the involved isomers and mechanisms are needed to provide a comprehensive mechanistic understanding.

Acknowledgements

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References


Table 1. Physicochemical properties and pan-HDAC inhibition activities

<table>
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<tr>
<th>Compound</th>
<th>Carbon linker (n)</th>
<th>HDAC IC₅₀ [nM]</th>
<th>PSA</th>
<th>MW</th>
<th>CLogP</th>
<th>MV(Å)³</th>
<th>Excitation (nm)</th>
<th>Emission (nm)</th>
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<td>1</td>
<td>287</td>
<td>213.7</td>
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<td>1037.4</td>
<td>320</td>
<td>500</td>
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<td>125</td>
<td>218.4</td>
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<td>1067.5</td>
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<td>500</td>
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<td>SAHA</td>
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<td>264.32</td>
<td>0.99</td>
<td>868.7</td>
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PSA = polar surface area; MW = molecular weight; CLogP = calculated log of the partition coefficient; MV = Molecular volume; Excitation = excitation wavelength; Emission = emission wavelength. Compound structures are shown in Figure 1A.
Table 2. HDAC isomer inhibition activity

<table>
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<th>Compound</th>
<th>1</th>
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<th>3</th>
<th>4</th>
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<th>8</th>
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<td>3</td>
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<td>0.13</td>
<td>0.027</td>
<td>0.99</td>
<td>2.74</td>
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HDAC Class I (1, 2, 3 and 8); Class II (4, 5, 6, 7, 9 and 10); Class IV (11)
Table 3. Effect of compound 2 on cell cycle distributions

<table>
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<tr>
<th>Compound</th>
<th>Cell line</th>
<th>Cell cycle distribution (%)</th>
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<td></td>
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<tr>
<td>Control</td>
<td>LNCaP</td>
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<tr>
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<td>Control</td>
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<td>2</td>
<td>PC3</td>
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<tr>
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*GI₅₀* of SAHA at 1.04 µM for PC3 and the compound 2 at 1.54 µM for PC3 and 1.3 µM for LNCap were used. A549 cells were treated with 10 µM of compound 2 and SAHA, no obvious change at 1 µM concentration was observed. MCF-7 cells were treated with 1 µM of compound 2 and SAHA.
**Figure Legends:**

**Figure 1.** A: Design of florescent hydroxamic acid HDAC inhibitors. B: Proposed binding model of 2 (green) in complex with HDAC6, 7 and 8. X-ray structures of HDAC7, and HDAC8 were used, whereas HDAC6 is a homology model based on HDAC7. Highlighted residues that make critical interactions with compound 2 are represented by the stick model (magenta). Hydrogen bonds are indicated by dotted line (black), zinc colored as orange and water colored as blue.

**Figure 2.** A: Co-localization of fluorescent compound 2 in PC-3 cells. Images were taken at 695 nm with a multiphoton laser. Cells were treated with 20 μM of compound 2 and images were obtained 60 minutes following drug exposure. a. Cytoplasm contains fluorescent compound 2 (Green). b. The nucleus is stained with propidium iodide (red). c. Differential interference contrast (DIC) image (grey). d. Merged image of a, b and c. B: Compound 2 effects on nuclear histone acetylation in A549 cells. Images were taken at 695 nm with a multiphoton laser. a. Acetylated histone (red); b. Propidium Iodide stain of the nucleus; c. DIC image; d. 20 μM of compound 2 (green) shown in cytoplasm; e. Fused image of a, b, c and d; f. Control acetylation. C: Effects of 2 on tubulin acetylation in the cytoplasm of A549 cells. Images were taken at 695 nm with a multiphoton laser. a. Acetylated tubulin (red). b. 20 μM of fluorescent compound 2 (green); c. DIC image; d. Merged image of a, b, and c; e. Control acetylated tubulin (without compound 2).

**Figure 3.** HDAC inhibitor trap HDAC4 in the cytoplasm of human prostate cancer cells. A. Untreated PC3 cells. B. PC3 cells treated with 1 μM of compound 2 for 24 hrs and detected for HDAC4. C. PC3 cells treated with 1 μM SAHA for 24 hrs and detected for HDAC4. Images were taken at 695 nm with a multiphoton laser.
Figure 4. A. p21 expression in PC3 cells. B. Acetylated tubulin expression in T24 cell lines. C. Acetylated histone 3 (H3) in PC3 cells when treated with compound 2.
Figure 1: Kong et al.

A.

Cap region \[\text{SAHA} \] Linker \[\text{Hydroxamate} \]

Fluorescent probe

General Scaffold

Dansyl group

Compounds: 1 \((n = 1)\); 2 \((n = 2)\); 3 \((n = 3)\); 4 \((n = 4)\)

B.

C.

D.
Figure 2: Kong et al.

A. a, b, c, d

B. a, b, c, d, e, f

C. a, b, c, d, e
Figure 3: Kong et al.

A. Untreated  DAPI  Overlap

B. Treated with 2  DAPI  Overlap

C. Treated with SAHA  DAPI  Overlap
Figure 4: Kong et al.

A. SAHA vs Compound 2 Control

- **β-actin**
- **p21**

B. SAHA vs Compound 2 Control

- Acetylated Tubulin
- **β-actin**

C. Control vs SAHA vs Compound 2

- **β-actin**
- Acetylated H3
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Yali Kong, Mira Jung, Kan Wang, et al.

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