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The structural requirements of histone deacetylase inhibitors: SAHA analogs modified at the C5 position display dual HDAC6/8 selectivity

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ABSTRACT

Histone deacetylase (HDAC) proteins have emerged as important targets for anti-cancer drugs, with four small molecules approved for use in the clinic. Suberoylanilide hydroxamic acid (Vorinostat, SAHA) was the first FDA-approved HDAC inhibitor for cancer treatment. However, SAHA inhibits most of the eleven HDAC isoforms. To understand the structural requirements of HDAC inhibitor selectivity and develop isoform selective HDAC inhibitors, SAHA analogs modified in the linker at the C5 position were synthesized and tested for potency and selectivity. C5-modified SAHA analogs displayed dual selectivity to HDAC6 and HDAC8 over HDAC 1, 2, and 3, with only a modest reduction in potency. These findings are consistent with prior work showing that modification of the linker region of SAHA can alter isoform selectivity. The observed HDAC6/8 selectivity of C5-modified SAHA analogs provide guidance toward development of isoform selective HDAC inhibitors and more effective anti-cancer drugs.

Histone deacetylase (HDAC) proteins have a prominent regulatory role in gene transcription and cell function. HDAC proteins catalyze the removal of the acetyl group from ε-acetyllysine residues on nucleosomal histones. Upon deacetylation of histone proteins, the interaction between histone proteins and DNA increases, which reduces DNA expression and gene transcription.[1,2] In addition, HDAC proteins affect intracellular interactions, protein localization, and protein stability through deacetylation of non-histone substrates.[3–6] Beyond basic cell biology, HDAC proteins are overexpressed in several cancers, as well as other diseases.[7]

HDAC proteins require either metal ions or NAD^+ as cofactors for catalysis.[8] The eleven metal-dependent HDAC isoforms are grouped as classes I, II, or IV, depending on their size, cellular localization, and homology to other HDAC proteins.[9] The seven NAD^+ dependent HDAC proteins are grouped as class III. The metal-dependent HDAC proteins comprise eleven isoforms (HDAC 1–11) and are the focus of this work.

Each metal-dependent HDAC isoform has been associated with various cancers. For example, HDAC1 was overexpressed in lung and colon cancers,[10,11] while HDAC2 displayed aberrant expression in colorectal and gastric cancer.[12] Abnormal activity of HDAC8 was observed in acute myeloid leukemia, T-cell lymphoma, and neuroblastoma.[13] HDAC6 was highly expressed in leukemia, ovarian cancer, and oral squamous cell carcinoma.[14–16] Moreover, HDAC6 plays an important role in cancer cell growth and survival through several non-epigenetic pathways.[17] Importantly, HDAC6 and HDAC8 were expressed at abnormally high levels in various human breast cancer cell lines and were associated with breast cancer invasiveness and metastasis, making them both interesting for anti-cancer drug design.[18]

Due to their fundamental role in cancer, inhibitors targeting HDAC proteins have been developed. HDAC inhibitors showed the ability to reduce proliferation and metastasis, and promote apoptosis in several cancers.[14,18–22] Four HDAC inhibitors have been approved by the FDA for cancer treatment.[20,23–26] For example, SAHA (suberoylamide hydroxamic acid, Vorinostat, Zolinza™) and Belinostat (PXD101, Belodaq™) (Fig. 1) were approved for treatment of T-cell lymphoma,[23–25] while Panobinostat (LBH-589, Farydak™) (Fig. 1) was approved for treatment of multiple myloma.[26] SAHA inhibits most of the eleven metal-dependent HDAC proteins, with only a modest selectivity against HDAC8.[27,28] The non-selectivity of SAHA might explain the side effects observed in clinic, but certainly limits the use of SAHA to study individual HDAC isoforms in cancer biology.[29] In the last four years, several dual HDAC6/HDAC8 selective inhibitors have been developed.[30–33] Dual inhibition of HDAC6 and HDAC8 can possibly have synergistic therapeutic applications in treatment of various cancers, which can improve anti-cancer efficiency compared to current non-selective HDAC inhibitors.[30–34]

SAHA and most of the HDAC inhibitors have a similar pharmacophore that consists of three structural elements (Fig. 1).

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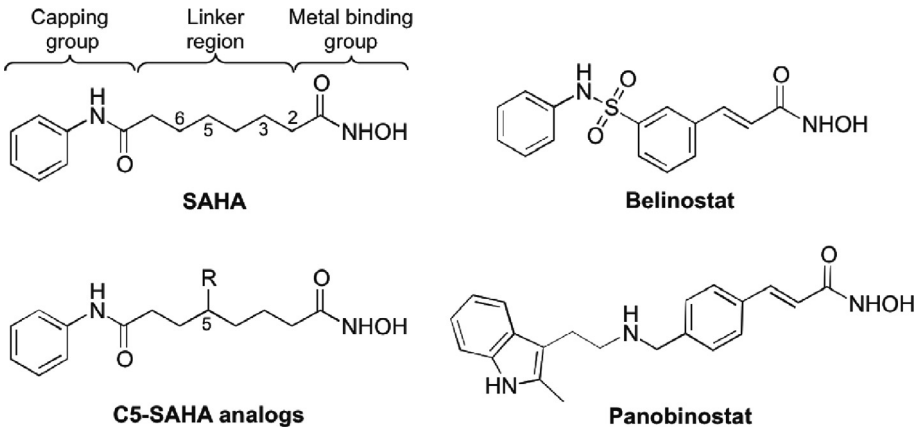


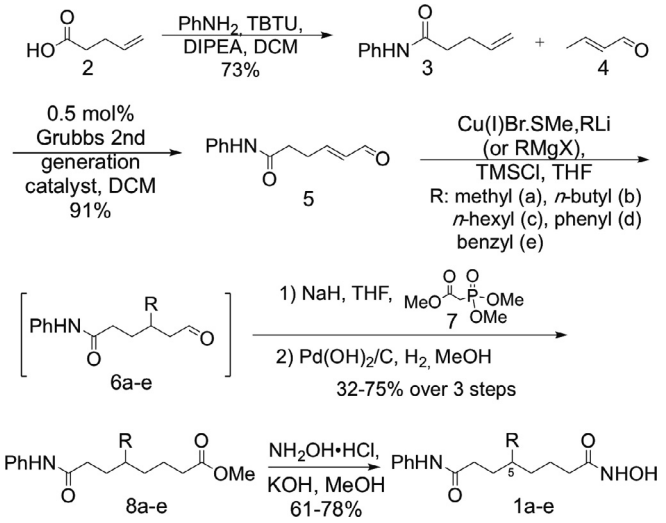
Fig. 1. Chemical structures of the FDA-approved drugs SAHA, Belinostat, and Panobinostat, along with the C5-modified SAHA analogs reported here.

The capping group interacts with solvent-exposed residues of the HDAC active site, while the metal binding group forms key interactions with the catalytic metal deeply buried in the active site (Fig. 1). The linker region that connects the capping and the metal binding groups is positioned in the narrow hydrophobic active site channel. Both the capping and the metal binding groups have been modified extensively in HDAC inhibitor design.[35–40] In contrast, few studies report modification of the linker region.[41–44] To study the effect of substitution on the linker region, SAHA analogs substituted at carbon 2 (C2), 3 (C3), or 6 (C6) of the linker region were synthesized and screened (Fig. 1). C2-hexyl SAHA displayed dual HDAC6/8 selectivity,[44] C3-ethyl SAHA showed HDAC6 selectivity,[42] and C6-butyl SAHA inhibited HDAC1 and 6 over HDAC3.[43] The conclusion of this prior work is that modification of the SAHA linker can alter inhibitor specificity.

Guided by this prior work, here we explored the effect of substituents on the C5 position of SAHA (Fig. 1). SAHA analogs substituted at the C5 were synthesized and tested for potency and selectivity both *in vitro* and *in cellulo*. Several analogs showed dual HDAC6/8 selectivity over HDAC1, 2, and 3, with a modest reduction in HDAC6 inhibition but enhanced HDAC8 inhibition compared to SAHA. This study documents that modifying the linker region of SAHA can alter its selectivity with minimal effect on potency.

Synthesis of C5-modified SAHA analogs

C5-modified SAHA analogs **1a**–**e** were synthesized as shown in Scheme 1. The synthesis started from a coupling reaction of 4-pentenoic acid **2** with aniline using TBTU to obtain amide **3**, which was then reacted with crotonaldehyde **4** via a cross metathesis reaction using second generation Grubbs' catalyst to afford the α,β-unsaturated aldehyde **5**. Aldehyde **5** was substituted with different groups through a 1,4-conjugate addition using organolithium or organomagnesium cuprates to yield intermediates **6a**–**e**. Horner–Wadsworth–Emmons olefination of **6a**–**e** with trimethyl phosphonoacetate followed by reduction gave amide esters **8a**–**e** with a saturated linker. Finally, amide esters **8a**–**e** were reacted with hydroxylamine to afford the C5-substituted SAHA derivatives **1a**–**e** as racemic mixtures.



Scheme 1. Synthesis of C5-modified SAHA analogs (**1a**-**e**).

Table 1
IC_{50} values for SAHA and C5-modified SAHA analogs (**1a**–**1e**) with HeLa cell lysates.[a]

Compounds	R	IC_{50} (μM)
SAHA	H	0.20 ± 0.02
1a	Methyl	0.10 ± 0.01
1b	*n*-Butyl	5.0 ± 0.4
1c	*n*-Hexyl	6.5 ± 0.1
1d	Phenyl	2.2 ± 0.1
1e	Benzyl	6.2 ± 0.2

[a] Mean IC_{50} value and standard error of at least three independent trials are shown (Fig. S52 and Table S1).

In vitro screening of C5-modified SAHA analogs

As a preliminary screen, the new analogs were tested for their global HDAC inhibition with HeLa cell lysates as the source of all HDAC proteins (Table 1). SAHA was also tested as the parent unsubstituted control molecule. The inhibitory activities of the analogs were measured with the HDAC-Glo™ I/II substrate (Promega). C5-methyl SAHA analog **1a** showed greater potency compared to SAHA (100 nM vs 200 nM IC_{50} values, Table 1). However, all other analogs showed weaker potency than SAHA (11- to 33-fold reduction in potency), with IC_{50} values from 2.2 to 6.5 μM (Table 1). The observed lower potencies of compounds **1b**–**1e** may be due to selectivity for specific HDAC isoform(s), which lowered the potency against lysates that contains all HDAC isoforms. The lower potency observed here was similar to what was observed with the C2-modified SAHA analogs.[44]

To test isoform selectivity, the parent molecule, SAHA, and all the C5-modified SAHA analogs were tested at a single concentration using the recently developed ELISA-based HDAC activity assay

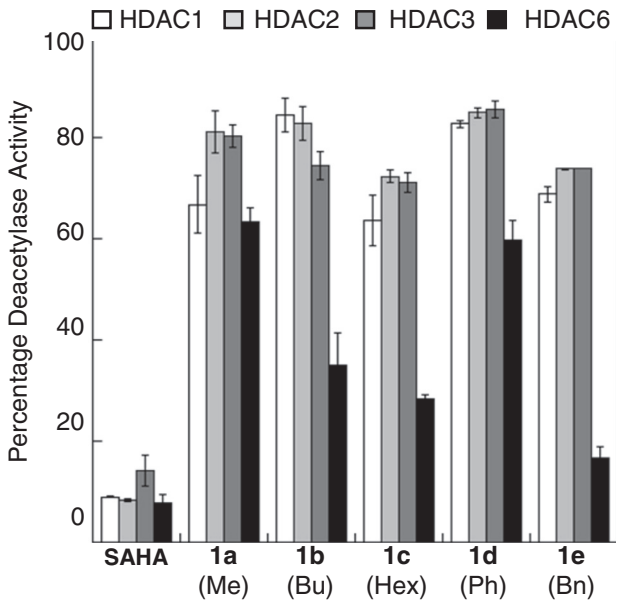


Fig. 2. *In vitro* isoform selectivity screening of C5-modified SAHA analogs (**1a**–**e**) against HDAC1, HDAC2, HDAC3, and HDAC6 using the ELISA-based HDAC activity assay. Analogs **1a**–**e** were tested at 0.025, 0.25, 1.25, 0.125, and 1.25 μM final concentrations, respectively. SAHA was tested at 1 μM concentration in a previous report using the same assay procedure.[28] Mean percent deacetylase activities from a minimum of two independent trials with standard errors were plotted (Table S2).

(Fig. 2).[28] SAHA, as expected, showed no selectivity among HDAC1, 2, 3, and 6.[28] Interestingly, several C5-SAHA analogs displayed more potent inhibition against HDAC6 compared to HDAC1, HDAC2, and HDAC3 (Fig. 2). The analogs that showed the greater difference in potency with HDAC6 versus the other isoforms were C5-*n*-butyl (**1b**), C5-*n*-hexyl (**1c**), and C5-benzyl (**1e**). The C5-methyl SAHA (**1a**) and C5-phenyl SAHA (**1d**) showed only a small difference in potency comparing HDAC6 to the others (Fig. 2).

IC_{50} values for the most selective derivatives **1b**, **1c**, and **1e** were determined with HDAC1, HDAC2, HDAC3, HDAC6, and HDAC8 isoforms to quantitatively assess the selectivity (Table 2). HDAC8 was also tested due to its similar active site compared to HDAC6. The IC_{50} values of SAHA as the parent compound were included as well (Table 2).[28] SAHA displayed similar IC_{50} values against HDAC1, 2, 3, and 6, with 6- to 27-fold selectivity against HDAC8.[28,44] Both C5-*n*-butyl (**1b**), and C5-*n*-hexyl (**1c**) SAHA analogs displayed modest selectivity, with 3- to 5-fold and 5- to 7-fold selectivities for HDAC6 and HDAC8 over HDAC1, 2, and 3 (Tables 2 and S6). In addition, **1b** and **1c** showed modest reductions in HDAC6 potency (IC_{50} values of 320 and 410 nM), but similar potency against HDAC8 (430 and 420 nM) compared to SAHA (Table 2). The most potent and selective analog was C5-benzyl SAHA (**1e**), which displayed 8- to 21-fold selectivity for HDAC6 and HDAC8 over HDAC1, 2, and 3 and IC_{50} values of 270 and 380 nM with HDAC6 and HDAC8, respectively (Tables 2 and S6). The selectivity was due to a dramatic reduction in potency with HDAC1, 2, and 3 (14- to 80-fold), but only a modest potency reduction with HDAC6 (8.5- to 12-fold) and similar potency with HDAC8 (380–540 nM), compared to SAHA. Modification of SAHA at the C5 position of the linker led to selectivity for HDAC6 and 8 over HDAC1, 2, and 3.

The size of the substituent had a substantial effect on selectivity (Fig. 2 and Table 2). More bulky or longer alkyl chain enhanced the selectivity to HDAC6 and 8 over HDAC1, 2, and 3. As suggested by previous reports,[44] larger substituents in the linker region might have led to enhanced HDAC6/8 selectivity due to the wider active site entrance of both HDAC6 and HDAC8 compared to HDAC1, 2 and 3. Steric clash with the active sites of HDAC1, 2, and 3 might explain the reduction in inhibition of HDAC1, 2, and 3 (36- to 290-fold reduction in IC_{50} values, see Table 2).[44,45] Consistent with this analysis, docking studies of the structurally similar C2-hexyl SAHA analog showed that both enantiomers had a similar selectivity and IC_{50} values, which suggests that the size of the substituent is the origin of the observed selectivity rather than the chirality of the substituted carbon.[44]

In cellulo selectivity testing

To test the analogs in a more biological context, the C5-benzyl (**1e**) SAHA analog was tested for selectivity in cells. The inhibition of HDAC6 was monitored by detecting the levels of its known substrate acetyl-α-tubulin (AcTub), whereas Class I HDAC (HDAC1, 2, and 3) inhibition was monitored by observing the known substrate, acetyl-histone 3 (AcH3). SAHA or C5-benzyl SAHA **1e** were incubated with U937 leukemia cells before lysis and western blot analysis of protein acetylation (Fig. 3). As expected, SAHA increased the levels of both acetyl-α-tubulin and acetyl-histone H3 to a similar extent (Fig. 3, lane 1), which is consistent with its non-selective inhibition of HDAC1, 2, 3, and 6 isoforms. On the

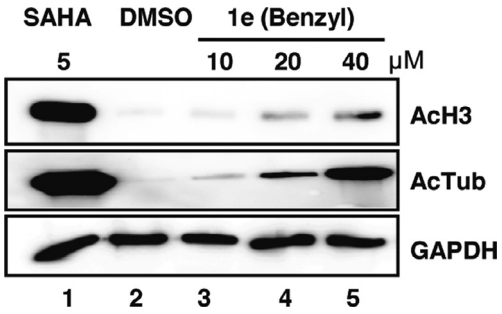


Fig. 3. Western blots analysis of acetyl-lysine 9 of histone H3 (AcH3) and acetyl-lysine 40 of α-tubulin (AcTub) after treatment with SAHA or C5-benzyl SAHA **1e**. U937 cells were treated with DMSO (1%), SAHA (5 μM), or increasing concentrations of C5-benzyl SAHA (**1e**) analog (10–40 μM), before lysis, SDS-PAGE separation, transfer to a PVDF membrane, and western analysis with AcH3 or AcTub antibodies. GAPDH levels in the samples were also probed as a gel load control. A DMSO control sample was included for comparison to inhibitor-treated samples. Repetitive trials are shown in Fig. S56.

Table 2
IC_{50} values for SAHA and C5-modified SAHA analogs **1b**, **1c**, and **1e** against HDAC1, 2, 3, 6 and 8.[a]

Compound	IC_{50} values (nM)				
	HDAC1	HDAC2	HDAC3	HDAC6	HDAC8
SAHA[b]	33 ± 1	96 ± 10	20 ± 1	33 ± 3	540 ± 10
1b (*n*-butyl)	1100 ± 100	1300 ± 100	1600 ± 100	320 ± 30	430 ± 10
1c (*n*-hexyl)	2100 ± 100	2500 ± 100	2900 ± 300	410 ± 60	420 ± 20
1e (benzyl)	2900 ± 300	3500 ± 100	5800 ± 800	270 ± 20	380 ± 20

[a] Mean IC_{50} value and standard error of at least three independent trials are shown (Figs. S53–S55 and Tables S3–S5).
[b] Previously reported IC_{50} values using the same assay procedure.[28]

other hand, C5-benzyl SAHA analog **1e** showed a dose dependent selective increase in levels of acetyl-α-tubulin, which was greater than the increased levels of acetyl histone H3 (Fig. 3, lanes 3–5) compared to the DMSO control (Fig. 3, lane 2). The observed HDAC6 selectivity of the C5-benzyl SAHA **1e** in cells is consistent with the selectivity observed in the *in vitro* screening (Table 2 and Fig. 3).

In vitro cancer cell growth inhibition

To evaluate the ability of the C5-modified SAHA analogs to influence cell growth, the most selective analogs were tested. C5-*n*-butyl (**1b**), C5-*n*-hexyl (**1c**), and C5-benzyl (**1e**) SAHA analogs were tested at 1 and 10 μM concentrations using MTT assay. Jurkat cells, a T-cell lymphoma derived cancer cell line, were selected due to the role of both HDAC6 and HDAC8 in lymphoma.[22,46] The tested analogs displayed cytotoxicity against the cell line, with 39%, 24%, and 50% cell viability with C5-*n*-butyl SAHA (**1b**), C5-*n*-hexyl SAHA (**1c**), and C5-benzyl SAHA (**1e**) at 10 μM concentrations, respectively (Fig. 4). On the other hand, SAHA demonstrated higher cytotoxicity than the C5-SAHA analogs, with 49% cell viability at 1 μM. The reduced cytotoxicity of the analogs compared to SAHA can be due to their lower potency (8- to 12-fold reduction in IC_{50} values with HDAC6 compared to SAHA, Table 2). In addition, the nonselective inhibition of most HDAC isoforms by SAHA might contribute to its higher cytotoxicity (Table 2 and Fig. 4).

In conclusion, C5-modified SAHA analogs displayed dual HDAC6/8 selectivity. The best compound was C5-benzyl SAHA (**1e**), which showed up to 21-fold selectivity for HDAC6 and 8 compared to HDAC1, 2, and 3, and IC_{50} values of 270 and 380 nM with HDAC6 and 8, respectively. C5-benzyl SAHA (**1e**) was tested for isoform selectivity in cells and showed selectivity consistent to what was observed *in vitro*. The fold selectivities observed with the C5-modified SAHA analogs were reduced compared to previously reported HDAC6/8-selective C2-modified SAHA analogs (49- to 300-fold selective for HDAC6 and 8 over HDAC1, 2, and 3 for the C2-hexyl SAHA).[44] On the other hand, the C5-modified SAHA analogs were more potent against HDAC6 and 8 (270–430 nM IC_{50} values) compared to C2-modifed SAHA analogs (600–2000 nM IC_{50} values for C2-hexyl SAHA). In general, this study and previous reports indicate that modifying SAHA in the linker region can alter the selectivity of HDAC inhibitors. In particular, the reduced potency of SAHA against HDAC8 compared to HDAC1, 2, and 3 was switched in the C5-modified analogs; C5-benzyl SAHA (**1e**) showed dramatically reduced potency for HDAC1, 2, and 3, but similar HDAC8 and modestly lower HDAC6 potency, compared to SAHA, which resulted in dual HDAC6/8 preference. HDAC6/8 dual selective inhibitors can be used as biological tools to study both HDAC6 and HDAC8-related cancer biology, and as leads for development of more effective anti-cancer agents targeting both HDAC6 and HDAC8.

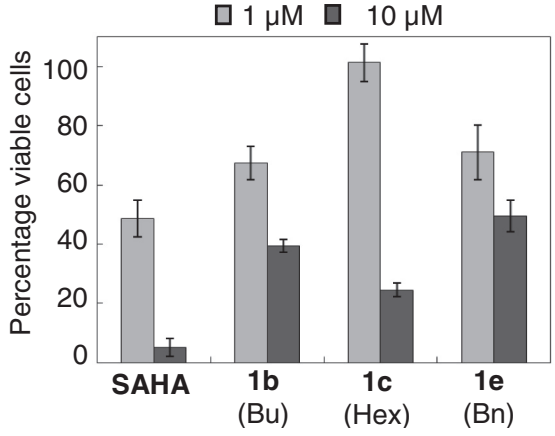


Fig. 4. Cytotoxicity screening of SAHA and C5-modified SAHA analogs **1b**, **1c**, and **1e**, at 1 and 10 μM concentrations using an MTT assay with the Jurkat cells. Mean percent cell viability from a minimum of three independent trials with standard errors were plotted (Table S7).

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.06.033.

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