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Brief Article

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Potent and Selective Inhibition of Histone Deacetylase 6 (HDAC6) Does Not Require a Surface-Binding Motif

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Supporting Information

ABSTRACT: Hydroxamic acids were designed, synthesized, and evaluated for their ability to selectively inhibit human histone deacetylase 6 (HDAC6). Several inhibitors, including compound **14** (BRD9757), exhibited excellent potency and selectivity despite the absence of a surface-binding motif. The binding of these highly efficient ligands for HDAC6 is rationalized via structure−activity relationships. These results demonstrate that high selectivity and potent inhibition of HDAC6 can be achieved through careful choice of linker element only.

INTRODUCTION

Histone deacetylases (HDACs) are enzymes responsible for catalyzing the hydrolysis of acetylated lysine residues located on histone as well as nonhistone proteins.[1] Such posttranslational modifications are crucial for the regulation of many cellular processes including gene transcription and protein function.[2] Inhibition of the 11 zinc-dependent human HDACs has proven to be a valuable strategy in the fight against cancer[3] and other human afflictions including psychiatric,[4] metabolic,[5] and infectious[6] diseases. These metal-dependent HDACs are divided into classes and subclasses: class I (HDACs 1, 2, 3 and 8), class IIa (HDACs 4, 5, 7, 9), class IIb (HDACs 6, 10), and class IV (HDAC11).[1c] The class IIb isoform HDAC6 is primarily localized in the cytosol and features two independently active catalytic domains.[2b,7] Functionally, HDAC6 is unique as the only zinc-dependent HDAC that controls α-tubulin acetylation.[7] In recent years, HDAC6 has been implicated in numerous disorders including several within the central nervous system (CNS).[8−10] Langley et al. reported that selective HDAC6 inhibition could promote the survival and regrowth of neurons following injury.[9] This finding was validated pharmacologically with tubastatin A, an HDAC6 selective compound, which was shown to confer protection in primary cortical neuron cultures under oxidative stress.[10]

Intrigued by the opportunity for pharmacological intervention in psychiatric diseases, we set out to identify selective small molecule inhibitors of HDAC6. A major challenge in CNS drug discovery is the efficient delivery of small molecules across the blood−brain barrier (BBB). Because the ability of a small molecule to cross the BBB is often inversely correlated with its size, we set out to define the essential pharmacophoric elements required for potent and selective binding of HDAC6. We utilized the concept of ligand efficiency (ligE), which is defined as biological activity per molecular size[11] and has become a beneficial tool in medicinal chemistry.[12] Compounds with high ligE (>0.50) often possess preferable lead-like characteristics for the CNS such as a low molecular weight (<350 g·mol^{-1}) and lower hydrophobicity (cLogP < 4).[13]

Many HDAC inhibitors are designed as structural mimics of acetyl-lysine and, as a result, often contain a zinc-binding group (ZBG), a linker, and a cap group (Figure 1).

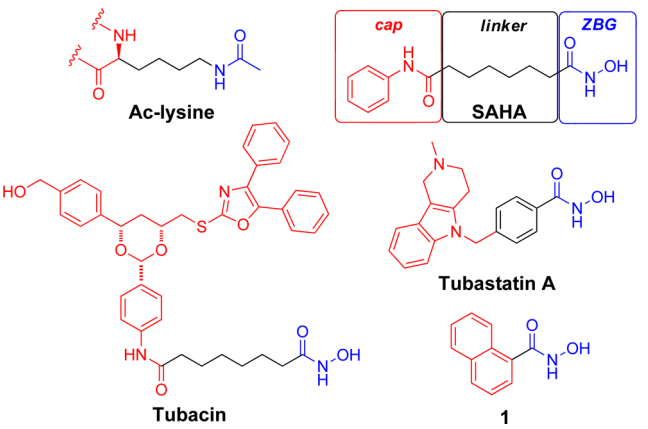

Figure 1. Structures of acetylated lysine and known HDAC inhibitors: SAHA, tubacin, tubastatin A, and "linkerless" compound **1**.

These pharmacophoric elements are exemplified by the structure of suberoylanilide hydroxamic acid (SAHA), a nonselective inhibitor of HDACs 1, 2, 3, 6, and 8, which possesses a 6-carbon alkyl chain linking a hydroxamic acid to a capping moiety. The hydroxamic acid zinc binding group and a large capping moiety are common features of selective HDAC6 inhibitors, and the latter is believed to be critical for selective inhibition.[14] Tubacin (Figure 1) was reported in 2003 by Haggarty et al. as the first selective HDAC6 inhibitor.[15] More recently, nonpeptidic macrocycles were reported by Auzzas et al. as HDAC6 selective inhibitors.[16] While structurally similar to SAHA in their ZBG and linker motifs, the selectivity was attributed to specific interactions between the unique capping

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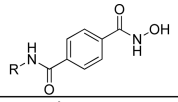
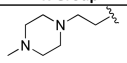
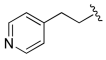
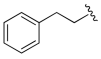
motif and the surface topology of HDAC6.[14b,16] While capping motifs can impart HDAC6 selectivity, the overall physicochemical properties and binding efficiency of these type of compounds suffer as reflected in a ligand efficiency of 0.16 for tubacin. Subsequently, a highly selective HDAC6 inhibitor, tubastatin A (Figure 1),[10] with an alternative linker motif, a substituted phenyl ring, was reported in 2010 by Butler et al. Tubastatin A also exploits interactions between the cap group and the HDAC enzyme rim and possesses a much more drug-like structure as exemplified by its higher ligE of 0.31. While these approaches focused on optimizing the surface binding motifs or cap to impart potency and selectivity for HDAC6, KrennHrubec et al. reported the design of "linkerless" or "capless" selective HDAC inhibitor such as compound **1**.[17] This compact (ligE of 0.38) HDAC8 selective inhibitor has >7-fold selectivity versus HDAC6 but displays weak inhibitory activity (14.0 μM). Similarly, for HDAC6, we chose to exploit close contacts and structural differences between the various isoforms within the catalytic binding domain. Herein, we describe our efforts to identify and characterize these critical linker interactions and to incorporate these design considerations into novel, selective, and highly efficient small molecule inhibitors of HDAC6.

RESULTS AND DISCUSSION

We began our studies with amide linked *para*-substituted phenyl hydroxamic acids (Table 1), as similar phenyl-linked compounds have been previously shown to inhibit HDAC6 selectively.[10,18] All compounds were tested against HDACs 1–9 in a trypsin-free microfluidic lab-on-a-chip assay.[19] IC_{50} values for HDAC2 and HDAC8 (representative of class I), HDAC4 (representative of class IIa), and HDAC6 are reported.

Table 1. IC_{50}s for HDACs 2, 4, 6, and 8



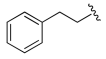
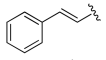
		HDAC Isoform Inhibition IC50 (μM)[a]			
Compound	R Group	HDAC 2	HDAC 4	HDAC6	HDAC8
2		16.79 ± 1.17	>33.33	1.36 ± 0.023	20.91 ± 0.765
3		0.341 ± 0.065	>33.33	0.008 ± 0.001	1.33 ± 0.452
4		0.607 ± 0.035	>33.33	0.004 ± 0.0001	1.15 ± 0.136
5		0.381 ± 0.006	>33.33	0.007 ± 0.0005	1.03 ± 0.122
6		0.321 ± 0.023	>33.33	0.037 ± 0.004	0.689 ± 0.018
7		1.61 ± 0.220	>33.33	0.028 ± 0.007	1.95 ± 0.106

[a]Values are the mean of two experiments. Data are shown as IC_{50} values in μM ± standard deviation. Compounds were tested in duplicate in a 12-point dose curve with 3-fold serial dilution starting from 33.33 μM.

Compound **2**, with an ethylene piperazine capping element, exhibited a 12-fold selectivity in favor of HDAC6 versus HDAC2, however, its potency for HDAC6 was modest (1.36 μM). The more lipophilic compound **3** (BRD8148)[20] proved to be a potent (8 nM) and selective (42-fold vs HDAC2) inhibitor of HDAC6; however, the pyridine nitrogen appears to have no effect as it did not confer improved potency or selectivity over the phenyl group in compound **4** (4 nM, 152-fold). Next, we probed the effect of the length of the surface group. While compounds **4**–**6** exhibited excellent potency for HDAC6, the extra carbon spacers in **4** only provided marginal improvement in selectivity for this isoform. In fact, the cap group only appeared to have a modest effect on the relative inhibition of HDACs 2, 4, 6, and 8. Strikingly, compound **7**, with a *para*-methyl amide, retained a high level of potency (28 nM) and selectivity (57-fold), demonstrating that a large capping group is not critical to achieve potent and selective inhibition of HDAC6.

Encouraged by the initial results with the *para*-substituted phenyl hydroxamic acids, we set out to identify the minimal pharmacophore that would confer potency and selectivity for HDAC6 (Table 2). To our surprise, capless phenyl hydroxamic acid, compound **8**,[21] exhibited good potency (115 nM) with a remarkable ligE of 0.69. Moreover, the selectivity of compound **8** versus HDAC2 (70-fold) and HDAC8 (17-fold) remained high.

Table 2. IC_{50}s for HDACs 2, 4, 6, and 8

		HDAC Isoform Inhibition IC50 (μM)[a]			
Compound	R Group	HDAC 2	HDAC 4	HDAC6	HDAC8
8		7.92 ± 0.096	>33.33	0.115 ± 0.013	1.92 ± 0.363
9		>33.33	>33.33	1.10 ± 0.076	11.62 ± 0.366
10		2.72 ± 0.427	>33.33	0.448 ± 0.001	2.35 ± 0.107
11		0.457 ± 0.013	19.46 ± 3.32	0.022 ± 0.0005	0.759 ± 0.034
12		1.05 ± 0.026	9.13 ± 0.256	0.012 ± 0.0005	0.431 ± 0.084
13		>33.33	>33.33	0.376 ± 0.058	3.72 ± 1.00
14		1.79 ± 0.165	21.80 ± 0.091	0.030 ± 0.006	1.09 ± 0.110

[a]Values are the mean of two experiments. Data are shown as IC_{50} values in μM ± standard deviation. Compounds were tested in duplicate in a 12-point dose curve with 3-fold serial dilution starting from 33.33 μM.

We next turned our attention toward small molecules containing a short linker between the ZBG and the aromatic group, as several reported HDAC inhibitors contain an alkyl or cinnamyl-linked hydroxamic acid motif.[21] The results (shown in Table 2, compounds **9**–**11**) demonstrate that an sp^2 carbon at the α-position of the hydroxamic acid is crucial for maintaining potent inhibition of HDAC6. Compounds **9** and **10**, which possess an alkyl linker between the ZBG and the aromatic group, demonstrated a reduced ability to inhibit HDAC6.

However, cinnamyl linked compound **11** exhibited increased potency for HDAC6, but the selectivity toward HDAC6 versus HDAC2 (21-fold), while still remarkable, suffered slightly as this compound became more potent on class I HDACs. The importance of the sp^2-hybridized carbon was further confirmed by the potency of cyclohexenyl compound **12** (12 nM) compared to the potency of cyclohexanyl compound **13** (376 nM). Moreover, compounds **11** and **12** illustrate that while the sp^2-carbon confers potency, substitution at the α-carbon enhances selectivity for the HDAC6 isoform.

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Table 3. HDAC Inhibitors' IC_{50}s for HDACs 1−9

	HDAC Isoform Inhibition, IC50 (μM)[a]								
Compound	HDAC 1	HDAC 2	HDAC 3	HDAC 4	HDAC 5	HDAC 6	HDAC 7	HDAC 8	HDAC 9
SAHA	0.004 ± 0.0001	0.011 ± 0.0005	0.003 ± 0.0005	>33.33	8.75 ± 1.80	0.002 ± 0.0002	>33.33	1.02 ± 0.187	>33.33
Tubastatin A	0.144 ± 0.051	0.360 ± 0.244	0.155 ± 0.037	1.10 ± 0.313	2.30 ± 0.46	0.002 ± 0.001	0.379 ± 0.277	0.681 ± 0.059	0.621 ± 0.132
8	4.73 ± 0.065	7.91 ± 0.096	7.83 ± 0.149	>33.33	>33.33	0.115 ± 0.013	15.60 ± 7.56	1.92 ± 0.363	>33.33
14	0.638 ± 0.064	1.79 ± 0.165	0.694 ± 0.033	21.80 ± 0.091	18.32 ± 5.31	0.030 ± 0.006	12.61 ± 4.49	1.09 ± 0.110	>33.33

[a]Values are the mean of two experiments. Data are shown as IC_{50} values in μM ± standard deviation. Compounds were tested in duplicate in a 12-point dose curve with 3-fold serial dilution starting from 33.33 μM.

To probe the selectivity of these compounds versus the broader family of HDAC isoforms, we profiled compounds **3**, **7**, **8**, **12**, and **14** against HDACs 1−9 (Table 3 and Supporting Information).[19] The cyclopentenyl linked compound **14** (BRD9757) represents one of the most concise inhibitors of HDAC6 reported to date, with an IC_{50} of 30 nM and excellent selectivity toward HDAC6 versus the class I (>20-fold) and class II (>400-fold) HDACs tested (Table 3). Consistent with this succinct design and remarkable potency, compound **14** displays a ligand efficiency of 0.84 for HDAC6. Potent and selective inhibition of HDAC6 does not require a surface-binding motif and can be achieved by relying on small linker motifs coupled with the hydroxamic acid ZBG.

To further validate the selectivity of these compounds, we investigated whether or not their biochemical selectivities translated in cellular functional assays. HeLa cells were treated with compounds at 10 and 30 μM for 24 h prior to measuring the acetylation status of α-tubulin (HDAC6 dependent)[7a] compared to total H3 acetylation (HDAC1, 2, 3 dependent)[1c] (Figure 2). As expected, we observed a dose-dependent

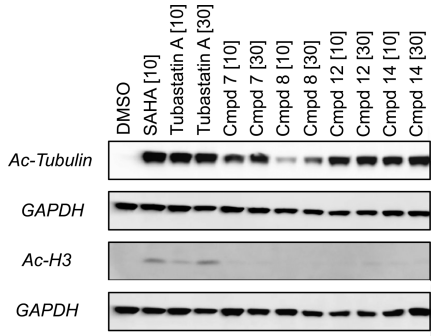


Figure 2. Treatment of HeLa cells for 24h with compounds **7**, **8**, **12**, and **14** results in the increase of Ac-tubulin but not Ac-H3. Concentrations in μM are shown in brackets.

increase in the level of Ac-tubulin when the cells were treated with compounds **7**, **8**, **12**, or **14** but did not observe a significant increase in Ac-H3 even at 30 μM. In contrast, SAHA and tubastatin A induced an increase in Ac-tubulin as well as a moderate increase in Ac-H3. This confirms that cellular HDAC6 is selectively inhibited by these highly ligand efficient capless hydroxamic acids in a robust manner.

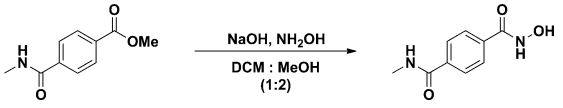
CONCLUSIONS

We demonstrated that large capping groups, such as those of tubacin and tubastatin A, are not necessary to achieve potent and selective inhibition of HDAC6. Several novel and highly efficient capless ligands were shown to have excellent potency and selectivity toward HDAC6 versus HDAC1−9. The selective inhibition of HDAC6 translated to a cell-based assay in which selective increase in the level of Ac-tubulin, without increasing histone acetylation, was observed upon treatment with capless HDAC6 inhibitors. Furthermore, we demonstrated that an sp^2-carbon at the α-position of the hydroxamic acid confers potency, while small capless cycloalkenyl motifs are sufficient to achieve high selectivity for the HDAC6 isoform. Ultimately, our studies should open the way for the design of selective small molecule HDAC inhibitors with optimized ligand efficiencies. These novel small molecule inhibitors can be used as tools for probing the biological functions and relevance of the different HDAC isoforms and serve as the basis for new selective inhibitors of other HDAC isoforms.

EXPERIMENTAL SECTION

See Supporting Information for details. All final compounds were confirmed to be of >95% purity based on HPLC LCMS analysis (Alliance 2795, Waters, Milford, MA). Purity was measured by UV absorbance at 210 nm. Identity was determined on a SQ mass spectrometer by positive and negative electrospray ionization. All reagents and solvents were purchased from commercial vendors and used as received. ^1H and ^{13}C NMR spectra were recorded on a Bruker 300 MHz or Varian UNITY INOVA 500 MHz spectrometer as indicated. Proton and carbon chemical shifts (δ) are reported in ppm relative to tetramethylsilane (δ 0 for both ^1H and ^{13}C) and DMSO-d_6 (^1H δ 2.50, ^{13}C δ 39.5). NMR data were collected at 25 °C. Flash chromatography was performed using 40−60 μm silica gel (60 Å mesh) on a Teledyne Isco Combiflash Rf system.

Representative Procedure for the Synthesis of Hydroxamic Acid Derivatives.



Methyl 4-(methylcarbamoyl)benzoate (0.20 g, 1.04 mmol) was dissolved in CH_2Cl_2 and methanol (1:2, 9 mL). The resulting solution was cooled to 0 °C, and hydroxylamine (50 wt % in water, 0.952 mL, 31.1 mmol, 30 equiv) was added, followed by sodium hydroxide (0.414 g, 10.35 mmol, 10.0 equiv). The reaction was allowed to warm to room temperature and stirred for 12 h. The solvent was then removed under reduced pressure, and the obtained solid was dissolved in water. The pH was adjusted to 7 with a 1N aqueous solution of HCl. The resulting precipitate was filtered and dried under high vacuum to afford *N*-hydroxy-*N*-methylterephthalamide (**7**) (90 mg, 45% yield) as a white solid. ESI+ MS: *m*/*z* 195.3 ($[M + H]^+$). ^1H NMR (300 MHz, DMSO-d_6): δ 11.33 (s, 1H), 9.14 (s, 1H), 8.55 (s, 1H), 7.95−7.75 (m, 4H), 2.79 (s, 3H). When analogues did not precipitate, after adjusting the pH, the water was removed under reduced pressure to afford white solids. The crude material was dissolved in cold ethanol and filtered. The obtained solids

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were then purified by reverse phase HPLC using a gradient of 0−20% acetonitrile in water (0.1% TFA).

■ ASSOCIATED CONTENT

Supporting Information

Spectroscopic characterization of the compounds in this paper; details related to biochemical and cellular studies. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

HDACs, histone deacetylases; CNS, central nervous system; BBB, blood−brain barrier; LigE, ligand efficiency; ZBG, zinc-binding group; SAHA, suberoylanilide hydroxamic acid; MD, molecular dynamics

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