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Inhibitors selective for HDAC6 in enzymes and cells

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ABSTRACT

Histone deacetylase inhibitors with anticancer or anti-inflammatory activity bind to Class I or Class I and II HDAC enzymes. Here we compare selectivity of inhibitors of a Class II HDAC enzyme (HDAC6) and find one that retains high selectivity in macrophages.

Acetylation of lysine residues in proteins is a reversible post-translational modification that impacts on many cellular processes, including signaling, RNA splicing, gene expression, protein stability and transport.[1] A recent proteomic analysis of three human cell lines identified 1750 nuclear and non-nuclear proteins modified by lysine acetylation at 3600 individual sites.[2] Lysine acetylation has been most extensively studied for histone proteins, where it regulates chromatin architecture and gene transcription.[1,3,4] An acetyl group is transferred to the ε-amino group of lysine residues in histones by enzymes called histone acetyl transferases (HATs) and removed by histone deacetylases (HDACs).

Inhibitors of HDAC enzymes have diverse effects on cell function, causing differentiation, growth inhibition, apoptosis and immunomodulation. Such pleiotropic effects may reflect distinct functions of specific HDAC enzymes, which fall into four classes based on sequence similarity and cofactor requirement. In humans, there are 18 HDAC genes, and 11 of these encode the classical (Classes I, II, IV) Zn^{2+}-dependent HDAC enzymes.[5] Class I enzymes (HDACs 1, 2, 3, 8) reside mainly in the nucleus,[6,7] Class IIa enzymes (HDACs 4, 5, 7, 9) translocate between the nucleus and cytoplasm in response to cellular signals,[4,8] while Class IIb enzymes (HDACs 6, 10) have two independent deacetylase domains and HDAC6 at least may be exclusively cytoplasmic.[9,10] Class III HDACs called Sirtuins (SIRT1–7) require NAD^+ for activity.[11–13] All other HDACs, including the only known human enzyme of Class IV, HDAC11,[14] are Zn^{2+}-dependent enzymes.[5]

Phenotypic analysis of $HDAC6^{-/-}$ mice suggests roles for this protein in bone homeostasis, immune functions and protection against neurodegenerative diseases.[15,16] At the cellular level, HDAC6 regulates autophagy,[16] motility[17] and adhesion.[18] Many synthetic HDAC inhibitors are known, but few are selective for HDAC6. TSA (**1**) and SAHA (**2**) are pan-inhibitors of HDACs,

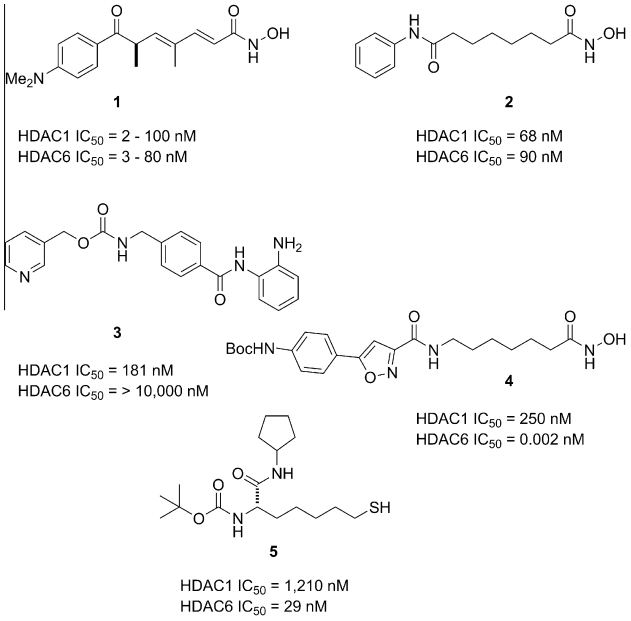

Figure 1. Relevant reported human HDAC inhibitors.[22–25]

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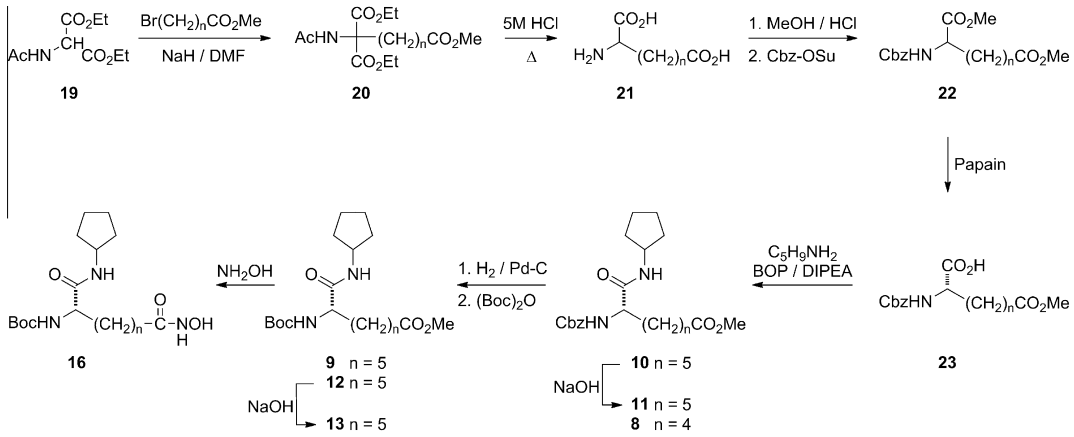


Scheme 1. Synthesis of carboxylic acid, ester and hydroxamate inhibitors.

MS275 (**3**) is selective for HDAC1, while hydroxamate **4** and thiol **5** are reportedly HDAC6 inhibitors (Fig. 1). Tubacin is known as a selective HDAC6 inhibitor but has poor drug like properties (mol wt 721), is not readily available and is difficult to synthesize.[19] Some HDAC inhibitors are anti-inflammatory,[20] **5** showing anti-inflammatory properties in mouse macrophages[21] but only at μM concentrations due to poor cell permeability and instability of the thiol group. Here we compare **5** and analogues for inhibition of recombinant human HDAC6 versus rh-HDAC1 enzymes and in human cells.

The synthesis of inhibitors **8**–**13** and **16** is shown in Scheme 1. Diethyl acetamidomalonate **19** was alkylated with methyl 5-bromopentanoate (or 6-bromohexanoate) giving **20** then hydrolysis with HCl and decarboxylation gave the racemic amino acid **21**. After esterification and Cbz protection, enzymatic resolution of **22** with papain[26] gave the (*S*)-amino acid **23**. Cyclopentylamine was coupled giving **10** then the Cbz group was cleaved by hydrogenolysis and replaced with Boc giving **9** and **12**. The side chain methyl ester was either hydrolyzed to give the acids **8**, **11** and **13** or converted to the hydroxamate **16** with hydroxylamine. Inhibitors with thiol (**5**, **14**) or alcohol (**6**, **7**, **15**) zinc binding groups were prepared by similar methods (Supplementary data) and the enantiopurity of the enzymatically resolved amino acids was confirmed by NMR analysis of diastereomeric derivatives (Supplementary data).

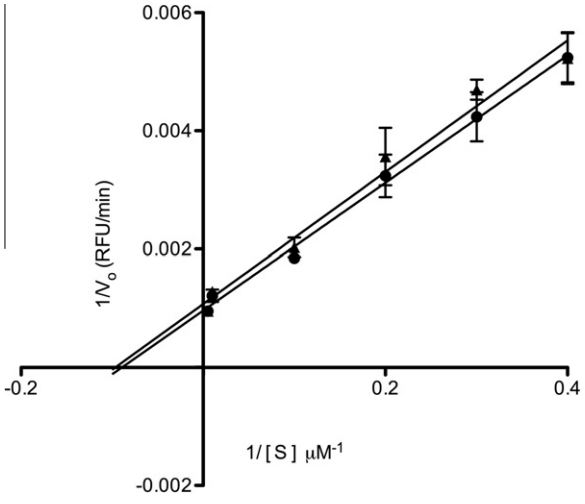
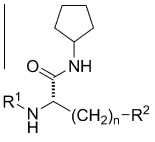


Figure 2. Lineweaver–Burk plot for HDAC processing of substrates. For HDAC6, Boc-Lys(Ac)-AMC (▲, K_M 20 μM) was a better substrate than *Z*-Lys(TFAc)-AMC, Boc-Lys(TFAc)-AMC and Ac-RGK(Ac)-AMC which all gave $K_M \geqslant 150$ μM. For HDAC1, Cbz-Lys(TFAc)-AMC (●, K_M 15 μM) was more effective than Boc-Lys(Ac)-AMC (K_M ~100 μM). Protein concentration was constant for all substrates (HDAC6, 0.35 ng/μL; HDAC1, 0.21 U/μL). Error bar = mean ± SEM (n = 3).

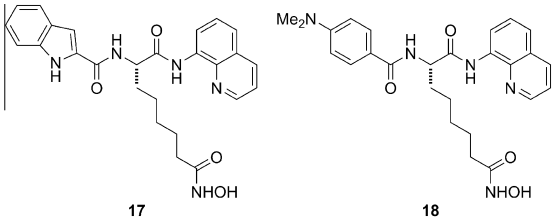
Table 1
Inhibitor potency for analogues of **5** at 1 μM against rh-HDAC6



Inhibitors	R^1	R^2	n	% Inhibition[a]
5	Boc	SH	5	98
6	Boc	OH	5	80
7	Boc	OH	4	50
8	Cbz	CO_2H	4	20
9	Boc	CO_2CH_3	4	40
10	Cbz	CO_2CH_3	5	65
11	Cbz	CO_2H	5	40
12	Boc	CO_2CH_3	5	60
13	Boc	CO_2H	5	45
14	Boc	SH	6	60
15	Boc	OH	6	3
16	Boc	CONHOH	5	99

[a] Assayed in triplicate in 25 mM Tris–HCl, pH 8, 37 °C.

Table 2
Comparative inhibition of rh-HDAC1 versus rh-HDAC6 enzymes



17 **18**

Inhibitor	IC_{50} ± SEM (nM) [a]			SI[b] HDAC1/HDAC6
	C log *P*	rh-HDAC1	rh-HDAC6	
1	2.06	10 ± 4	56 ± 1	0.2
2	0.98	76 ± 3	85 ± 5	0.9
3	−0.11	44 ± 4	>10,000	0.004
4	2.41	340 ± 2	40 ± 3	8.5
5	3.53	1731 ± 18	54 ± 2	32
6	2.12	>10,000	240 ± 10	>40
16	1.23	1420 ± 10	26 ± 2	55
17	2.56	48 ± 2	107 ± 1	0.4
18	2.86	21 ± 2	943 ± 6	0.02

[a] Compounds assayed in triplicate in 25 mM Tris–HCl (pH 8, 37 °C).
[b] SI = selectivity index.

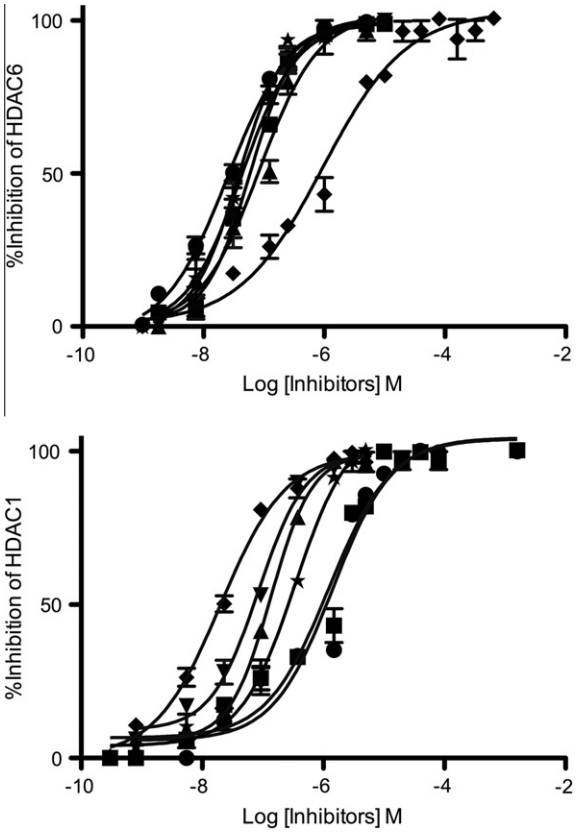


Figure 3. Concentration–response curves for inhibitors **1** (▼), **2** (▲), **4** (∗), **5** (■), **16** (●), **17** (♦) against rh-HDAC6 and rh-HDAC1. Error bar = mean ± SEM for three independent experiments.

A variety of different assays have been reported in the literature to determine HDAC activity[27] thus making a comparison of inhibitors difficult. No standard substrates have been adopted, different concentrations of enzyme/substrate are often used and K_M is usually not reported. Commercial assay kits often do not disclose the substrate or reagents. We evaluated several HDAC1 and HDAC6 substrates (Supplementary data) before standardizing assays (Fig. 2), to allow reliable potency/selectivity comparisons between inhibitors to be made (Table 2).

Thiol **5** was reported as a selective HDAC6 inhibitor (IC_{50} 29 nM),[25] and we found comparable potency (IC_{50} 54 nM) and 32-fold selectivity over rh-HDAC1 in our in vitro assay. HDAC6 inhibition translates to hyperacetylation of α-tubulin in cells, and was induced in mouse macrophages by **5** at >1 μM concentrations.[21] To improve potency at the cellular level, we made derivatives of **5** in which the N-terminal capping group (R^1), the Zn^{2+}-binding group (R^2) and/or linker length (n) were varied (**6–16**, Scheme 1, Tables 1 and 2).

The optimal linker length (n) was consistently found to be five methylenes regardless of the Zn^{2+}-binding group (Table 1). We had considered it possible that the six methylenes present in the alcohol **15** may better mimic the linker length of the thiol **5** than the shorter chain alcohol **6** by compensating for the significantly longer carbon–sulfur bond length but this was not so (Table 1). Similarly we thought that the carboxylic acid/esters **8** and **9** with four methylenes plus the carboxyl carbon may constitute a sufficiently long linker, however, this series also benefited from the longer five methylene linker. With the optimized linker of five methylenes, the thiol zinc binding group **5** conferred greater potency than alcohol **6**, the methyl esters (**10** and **12**) followed by the carboxylic acids (**11** and **13**) were less active. Replacing Boc with Cbz at the N-terminus (R^1) had little effect on potency.

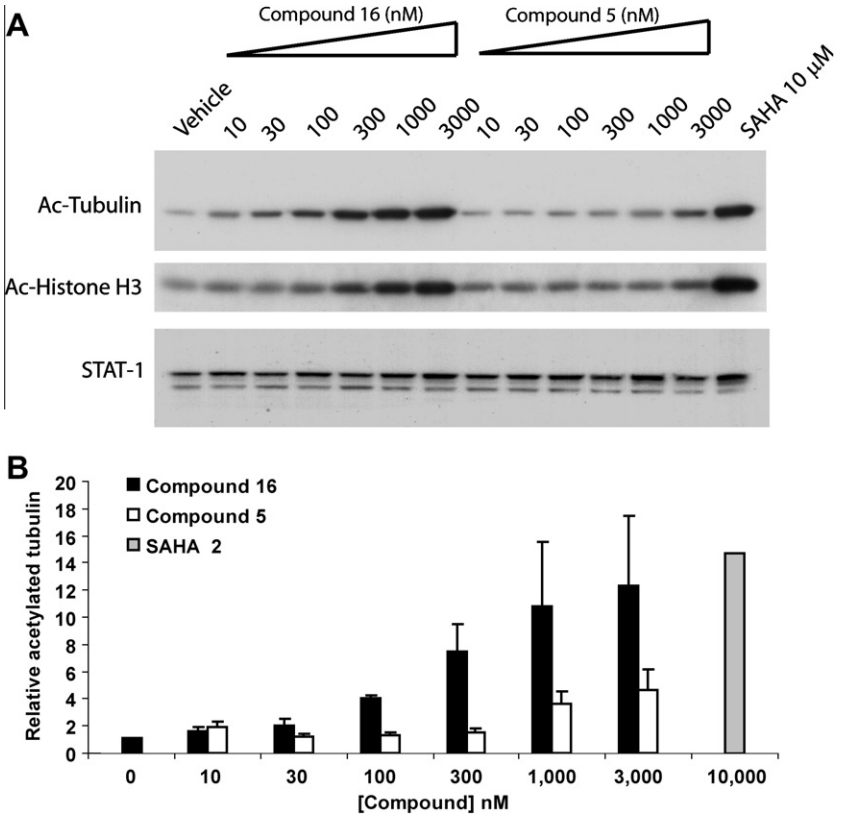


Figure 4. (A) Comparison of compounds **2**, **5** and **16** in PMA-differentiated THP-1 cells after 4 h treatment. Cell lysates were immunoblotted using antibodies against acetylated alpha-tubulin, acetylated histone H3 and STAT-1 (n = 2 experiments). (B) Semi-quantitative analysis (densitometry) of data from **A**.

Finally the hydroxamic acid **16** was found to be the most potent and selective inhibitor of this series. We were initially concerned that introduction of such a high affinity ligand for Zn^{2+} may result in a reduction of selectivity for HDAC6 or even give rise to a pan-inhibitor analogous to hydroxamates **1** and **2**. For example, the structurally related compound **17**, a potent HDAC inhibitor with anticancer activity[28] does not discriminate between HDAC6 and HDAC1 (IC_{50} 107 nM, HDAC6; 48 nM, HDAC1).

Furthermore, hydroxamate **18** showed more potent (45×) inhibition of rh-HDAC1 than rh-HDAC6 (Table 2). Suzuki et al. previously noted that loss of selectivity for HDAC6 resulted when the aliphatic cyclopentane ring of **5** was replaced by an aromatic ring.[25] Therefore, the combined effects of both Boc and cyclopentyl groups for HDAC6 selectivity together with the potency gains afforded by the hydroxamate group have synergised giving **16** with optimal HDAC6 potency and selectivity; IC_{50} 26 nM (rh-HDAC6) versus 1420 nM (rh-HDAC1) corresponding to 55-fold selectivity for rh-HDAC6 over rh-HDAC1 (Table 2, Fig. 3).

Since HDAC6 and HDAC1 deacetylate alpha-tubulin and histone H3, respectively,[10,15,29] comparative hyperacetylation of these protein substrates can be used to assess HDAC6 and HDAC1 inhibition in cells. Using these readouts, we have previously found some inhibitor selectivity for **5** at HDAC6 over HDAC1 in primary mouse macrophages.[21] Here we compare compounds **4**, **5** and **16** in PMA-differentiated THP-1 human macrophages. Replacement of the thiol in **5** with hydroxamate **16** greatly enhanced inhibition of HDAC6 in cells (>10-fold, Fig. 4), and **16** selectively inhibited HDAC6 since ~10-fold lower concentrations were required for tubulin hyperacetylation than for histone H3 hyperacetylation. Compound **5** did not display any selectivity for HDAC6 over HDAC1 in human PMA-differentiated THP-1 cells (Fig. 4), in contrast to our finding in mouse macrophages.[21] Compound **4** displayed similar potency to compound **16**, but had only modest selectivity in cells (Supplementary data). Thus, **16** may be a valuable *in vivo* probe for HDAC6 function.

In conclusion, we identify **16** as the most potent and selective synthetic inhibitor known for the recombinant human HDAC6 enzyme[†] and in human macrophages, important mediators of chronic inflammatory diseases.

Acknowledgments

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Supplementary data

Supplementary data associated (experimental methods, synthesis/modeling, enzyme/cell assays) with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.100.

[†] Compound **4** was reported as HDAC6-selective with pM,[23] then nM,[30] inhibitory potency. We re-examined an analogue (compound **1** in Ref. 30 Supplementary data **34**) in our assay and find IC_{50} 150 nM (HDAC6) versus 2980 nM (HDAC1) or 20-fold selectivity (Supplementary data). Using the substrate Ac-RHKK(Ac)-ACC as used in Ref. 30, we find their compound **1** to have IC_{50} 41 nM (HDAC6) versus 3000 nM (HDAC1) or 75-fold selective, whereas **16** gave IC_{50} 27 nM (HDAC6) versus 3567 nM (HDAC1) or 132-fold selectivity (Supplementary data). Thus, we are confident that **16** is more HDAC6-selective.

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