Highly Potent and Selective Histone Deacetylase 6 Inhibitors Designed Based on a Small-Molecular Substrate

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Abstract: To find novel histone deacetylase 6 (HDAC6)-selective inhibitors and clarify the structural requirements for HDAC6-selective inhibition, we prepared thiolate analogues designed based on the structure of an HDAC6-selective substrate and evaluated the histone/α-tubulin acetylation selectivity by Western blot analysis. Aliphatic compounds **17b−20b** selectively caused α-tubulin acetylation over histone H4 acetylation. In enzyme assays using HDAC1, HDAC4, and HDAC6, compounds **17a−19a** exhibited HDAC6-selective inhibition over HDAC1 and HDAC4.

Introduction

Histone deacetylases[a] (HDACs) are responsible for the deacetylation of the acetylated lysine residues in the N-terminal region of the core histones and are involved in transcriptional regulation, cell cycle progression, and developmental events.[1] Thus far, eighteen HDAC family members have been identified, and they are divided into two categories, i.e., zinc-dependent enzymes (HDAC1−11) and NAD+-dependent enzymes (SIRT1−7).[1a,2] HDAC6, a zinc-dependent HDAC isoform, is unique in that it is cytoplasmic and participates in the deacetylation of nonhistone proteins, such as α-tubulin and HSP90, as well as regulating important biological processes including microtubule stability and function, and molecular chaperon activity.[3] In addition, it has recently been reported that inhibition of HDAC6 has an antitumor effect in multiple myeloma cells.[4] Thus, HDAC6-selective inhibitors are of interest not only as tools for elucidating the more intricate biological functions of HDAC6, but also as candidate antitumor agents.

A number of structurally diverse HDAC inhibitors have been identified,[5] including **1** (trichostatin A, TSA),[6] **2** (suberoylanilide hydroxamic acid, SAHA),[7] **3** (CHAP31),[8] **4** (trapoxin B, TPX B),[6b,9] and **5** (MS-275)[10] (Chart 1). Most hydroxamates, such as **1** and **2**, inhibit all of the HDAC isoforms, whereas most non-hydroxamates, such as **4** and **5**, do not inhibit HDAC6.[3b,c,11] To date, the only known HDAC6-selective inhibitor is **6** (tubacin) (Chart 1), which was discovered using a combinatorial approach.[12] At present, there is little information about the structural requirements for HDAC6-selective inhibition. There-
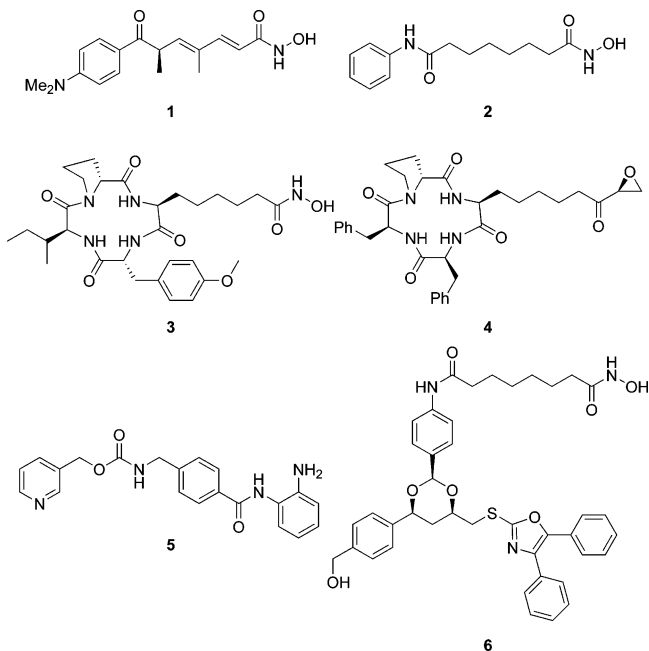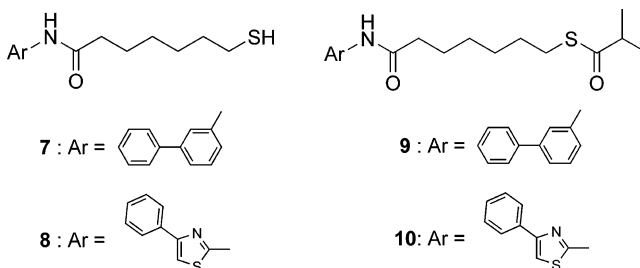
Chart 1. Examples of HDAC Inhibitors

Chart 2. Thiolate HDAC Inhibitors

fore, there is a need to develop novel HDAC6-selective inhibitors and then to study their structure−activity relationships.

We recently described a series of thiol-based analogues, including **7** (NCH-26) and **8** (NCH-31) (Chart 2), which act as novel non-hydroxamate HDAC inhibitors.[13] Thiols are thought to inhibit HDACs by coordinating the zinc ion which is required for deacetylation of the acetylated lysine substrate. Further, the *S*-isobutyryl prodrugs **9** (NCH-47) and **10** (NCH-51) (Chart 2), which are thought to be hydrolyzed to the free thiols within cells, showed potent cancer cell growth-inhibitory activities.[14] Following these findings, we performed further investigation of thiolate analogues, seeking to find HDAC6-selective inhibitors. We describe here the HDAC6 selectivity of thiolates whose designs were based on the structure of a small-molecular HDAC6-selective substrate.

Results and Discussion

Since HDAC6 has been reported as an α-tubulin deacetylase,[3b,3c] inhibition of HDAC6 and that of other HDACs can be assessed according to the accumulation of acetylated α-tubulin and acetylated histones, respectively, using Western blot analysis. We initially examined the histone/α-tubulin acetylation selectivity of **2**, **9**, and **10** (Figure 1). As reported previously,[11b] **2** caused the accumulation of both acetylated histone H4 and acetylated α-tubulin. Like other non-
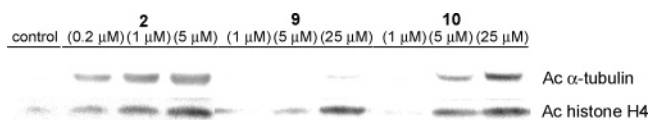

Figure 1. Western blot detection of acetylated α-tubulin and histone H4 levels in HCT116 cells after incubation with compounds **2**, **9**, and **10** for 8 h.
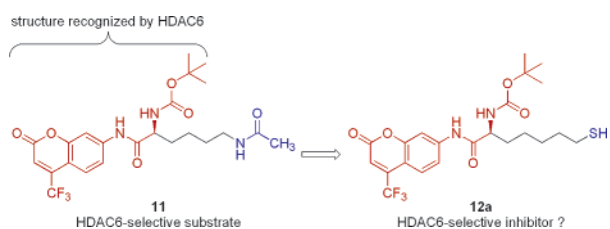

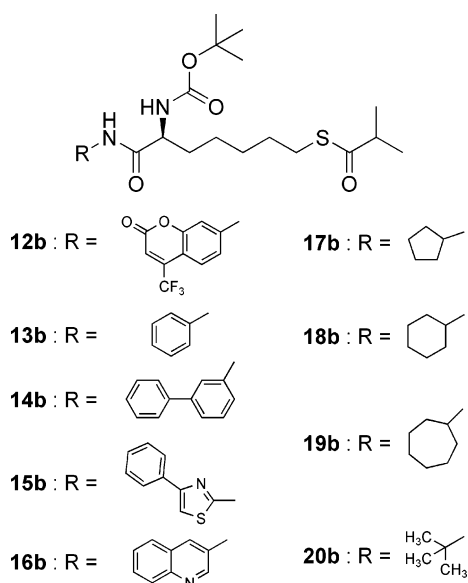**Figure 2.** Design of HDAC6-selective inhibitors.

Figure 3. Structures of the thioesters **12b**−**20b**.

hydroxamate HDAC inhibitors, compound **9** selectively caused histone H4 hyperacetylation, which indicates that compound **7** selectively inhibits nuclear HDACs over cytoplasmic HDAC6. However, unlike other non-hydroxamates, compound **10** increased the acetylation state of both histone H4 and α-tubulin. These results suggested that HDAC6-selective inhibitors might be obtained by structural modification of thiolate HDAC inhibitors.

In designing novel HDAC6-selective inhibitors, we focused initially on a small-molecular HDAC6-selective substrate **11**[15] (Figure 2). Jung and co-workers found that compound **11** is selectively deacetylated by HDAC6 in preference to HDAC1 and HDAC3. This indicated that the structure of *N*-Boc and trifluoromethyl coumaryl amide of compound **11** is selectively recognized by HDAC6, and so we considered that compound **12a**, in which the acetamide of **11** is replaced by a thiol, might behave as an HDAC6-selective inhibitor (Figure 2).

Since we used a cellular assay as the first screening, compound **12b** (Figure 3), the *S*-isobutyryl prodrug of compound **12a**, and its derivatives **13b**−**20b** were initially prepared. The route used for synthesis of compounds **12b**−**20b** is shown in Scheme 1. (*S*)-2-Amino-7-bromoheptanoic acid **21**[16] was treated with (Boc)$_2$O to give *N*-Boc compound **22**. The condensation of carboxylic acid **22** with an appropriate amine afforded amides **23**. Bromides **23** were treated with thioiso-

Scheme 1[a]
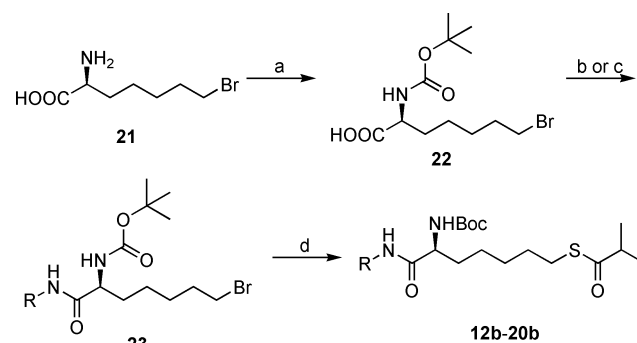
[a] Reagents: (a) (Boc)$_2$O, Et$_3$N, THF, H$_2$O, rt, 96%; (b) ArNH$_2$, POCl$_3$, pyridine, −15 °C, 10−48%; (c) RNH$_2$, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate, 1-hydroxybenzotriazole hydrate, Et$_3$N, DMF, rt, 35−63%; (d) thioisobutyric acid, Et$_3$N, EtOH, rt, 19−58%.
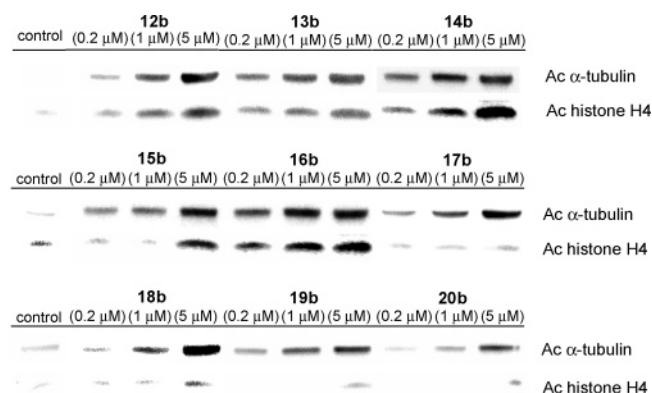

Figure 4. Western blot detection of acetylated α-tubulin and histone H4 levels in HCT116 cells after 8 h treatment with **12b**−**20b**.

butyric acid under alkaline conditions to yield the desired thioesters **12b**−**20b**.

We initially evaluated compound **12b** for the accumulation of acetylated α-tubulin and histone H4 using Western blot analysis (Figure 4). Although compound **12b** produced an increase in the accumulation of acetylated α-tubulin as compared with **9** and **10** (Figure 1), the selectivity was insufficient. In an attempt to improve the selectivity of the histone/α-tubulin acetylation, we decided to carry out the structural conversion of compound **12b**. The coumarin structure derived from a substrate for fluorescent enzyme assays was replaced with various aromatic (**13b**−**16b**) or aliphatic (**17b**−**20b**) moieties (Figure 3). Interestingly, although the aromatic compounds **13b**−**16b** did not show high selectivity, the aliphatic compounds **17b**−**20b** produced a dose-dependent increase of α-tubulin acetylation without a major increase in acetylated histone H4 (Figure 4). These results indicated that the aliphatic compounds **17b**−**20b** selectively inhibit HDAC6 in preference to other HDACs in cells. To quantify the selectivity of cyclopentyl **17b**, one of the most active α-tubulin acetylating agents in this series, acetylated α-tubulin and histone H4, were measured over a range of concentrations (Figure 5). The estimated EC$_{50}$ values of cyclopentyl **17b** for α-tubulin acetylation and histone H4 acetylation were 0.23 μM and >32 μM, respectively, and the selectivity index (histone acetylation EC$_{50}$/α-tubulin acetylation EC$_{50}$) was >140 which exceeded those of **2** (SI = 2.0) and **6** (SI = 75)[11b] (Figure 5 and Figure 1S of Supporting Information).

To confirm the HDAC6-selectivity of these aliphatic compounds, we performed in vitro enzyme assays. For the enzyme assays, we synthesized compounds **17a**−**19a**, the corresponding thiols of **17b**−**19b**. Compounds **17a**−**19a** were prepared by the
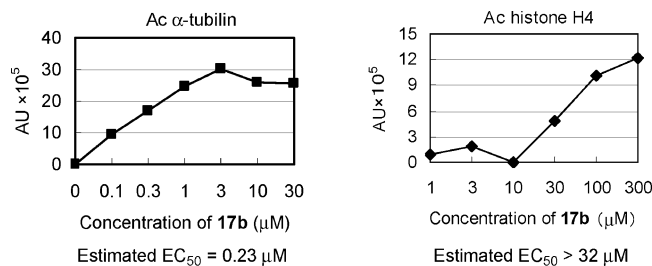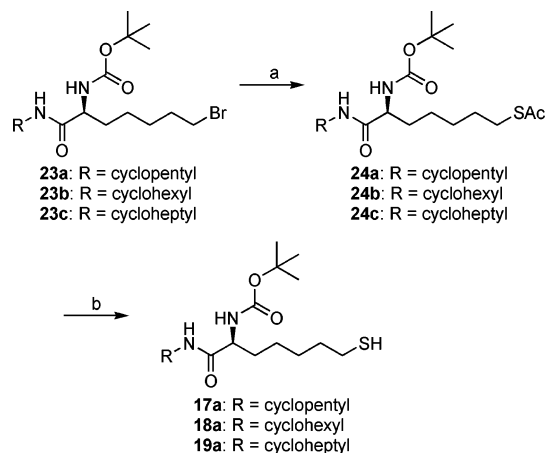
Figure 5. Quantification of acetylated α-tubulin and histone H4 levels in HCT116 cells treated for 8 h with **17b**. Compound **17b** was insoluble in 0.1% DMSO−McCoy5A culture medium at concentrations >300 μM.

Scheme 2[a]

23a: R = cyclopentyl
23b: R = cyclohexyl
23c: R = cycloheptyl

24a: R = cyclopentyl
24b: R = cyclohexyl
24c: R = cycloheptyl

17a: R = cyclopentyl
18a: R = cyclohexyl
19a: R = cycloheptyl

[a] Reagents: (a) KSAc, EtOH, rt, 75−93%; (b) NaOH, H_2O, EtOH, rt, 71−77%.

Table 1. In Vitro HDAC1-, HDAC4-, and HDAC6-Inhibitory Activities of **17a**−**19a**[a]

	IC_{50} (nM)			selectivity	
compd	HDAC1	HDAC4	HDAC6	HDAC1/ HDAC6	HDAC4/ HDAC6
1	21	34	81	0.26	0.42
6	ND[c]	ND	ND	4[b]	4[b]
7a	1210	1030	29	42	36
8a	1270	1140	36	35	32
9a	900	840	23	39	37

[a] Values are means of at least three experiments. [b] Data taken from the literature (ref 17). [c] ND = No data.

procedure outlined in Scheme 2. Bromides **23** were treated with potassium thioacetate to give compounds **24**, after which hydrolysis of the thioesters under alkaline conditions gave the desired thiols **17a**−**19a**.

The results of enzyme assays are shown in Table 1. The HDAC6-inhibitory activity of compounds **17a**−**19a** was greater than that of **1** (IC_{50} of **1** 81 nM, **17a** 29 nM, **18a** 36 nM, **19a** 23 nM). Furthermore, while **1** inhibited HDAC1 and HDAC4 rather than HDAC6 (HDAC1 IC_{50}/HDAC6 IC_{50} = 0.26; HDAC4 IC_{50}/HDAC6 IC_{50} = 0.42), compounds **17a**−**19a** excellently inhibited HDAC6 in preference to HDAC1 and HDAC4 (HDAC1 IC_{50}/HDAC6 IC_{50} = 35−42; HDAC4 IC_{50}/HDAC6 IC_{50} = 32−37). The HDAC6 selectivity of compounds **17a**−**19a** is about 10 times higher than that of **6** which showed about only 4-fold selectivity for HDAC6 over HDAC1 and HDAC6 in enzyme assays.[17] These enzyme assays revealed that compounds **17a**−**19a** are potent and selective inhibitors of HDAC6. The reason that there is essentially no difference in the activity and selectivity of compounds **17a**−**19a** is unclear, but it is assumable that HDAC6 has a hydrophobic pocket where

some sterically bulky alkyl groups can be placed and other HDAC isoforms do not have such a pocket.

In conclusion, we have identified novel HDAC6-selective inhibitors whose designs were based on the structure of the HDAC6-selective substrate **11**. As far as we could determine, they are the first inhibitors that show significant HDAC6-selective inhibition in both cellular and enzyme assays. We have also established that the presence of a bulky alkyl group in these compounds is important for HDAC6-selective inhibition. The structures of the newly discovered HDAC6-selective inhibitors **17**−**20** are simpler than that of **6** and appear to be suitable as lead structures for the further development of superior HDAC6-selective inhibitors. These findings provide a basis for constructing new tools for probing the biology of HDAC6 and for finding new candidate antitumor agents with potentially fewer side effects.

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