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Structural requirements of HDAC inhibitors: SAHA analogs functionalized adjacent to the hydroxamic acid

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Abstract—Inhibitors of histone deacetylase (HDAC) proteins such as suberoylanilide hydroxamic acid (SAHA) have emerged as effective therapeutic anti-cancer agents. To better understand the structural requirements of HDAC inhibitors, a small molecule library with a variety of substituents attached adjacent to the metal binding hydroxamic acid of SAHA was synthesized. The presence of a substituent adjacent to the hydroxamic acid led to an 800- to 5000-fold decrease in inhibition compared to SAHA. The observed results have implications for drug design, suggesting that HDAC inhibitors with substituents near the metal binding moiety will have inhibitory activities in the micromolar rather than nanomolar range.

Suberoylanilide hydroxamic acid (SAHA, Vorinostat, Zolinza™) recently gained FDA approval for the treatment of advanced cutaneous T-cell lymphoma (CTCL).[1] SAHA is an inhibitor of histone deacetylase (HDAC) proteins, which are linked to a variety of cancers.[2] While SAHA is the first HDAC inhibitor (HDACi) to meet FDA approval, several other small molecules that inhibit HDAC proteins are currently in clinical trials for cancer treatment.[3] Distinguishing characteristics of HDAC inhibitors include a metal binding moiety, a carbon linker, and a capping group (Fig. 1). Based on crystallographic analyses, the capping group is solvent-exposed and interacts with amino acids near the entrance of the active site, while the metal binding moiety resides in the protein interior and complexes the metal ion involved in catalysis.[4–6] The linker serves to position the capping and metal binding groups appropriately for high-affinity interactions with proteins. With a modular framework and application toward cancer treatment, HDAC inhibitors are viable targets for future drug design.

Previous HDACi design has emphasized modification of the capping group and the metal binding moiety. In the case of the metal binding moiety, SAHA contains a hydroxamic acid while other inhibitors contain thiols, epoxides, carboxylates, or benzamides.[7,8] For example, two HDAC inhibitors in clinical trials, MS-275 and valproic acid (Fig. 1), contain benzamide and carboxylate metal binding moieties, respectively,[9,10] and display IC_{50} values of 2 and 400 μM.[11,12] The reduced inhibitory activities compared to SAHA (110–370 nM IC_{50})[13,14] are partially explained by the presence of the benzamide or carboxylic acid group.[7,15,16] Interestingly, MS-275 displays modest preference toward select proteins within the 11-membered HDAC family.[17] Selective HDAC inhibitors would aid in elucidating the role of each individual HDAC protein in cancer and have the potential to be better drugs.[18] However, strictly selective HDAC inhibitors have yet to be discovered.

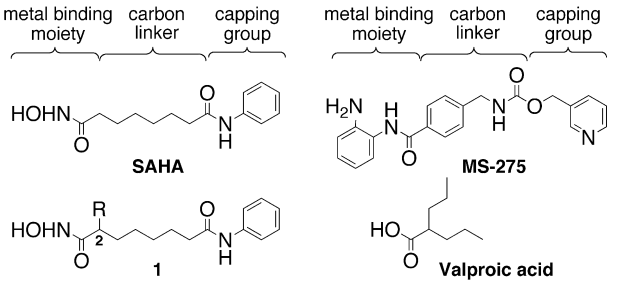

Figure 1. Structures of select HDAC inhibitors.

In addition to altering the metal binding moiety toward HDACi design, the hydrocarbon linker has been diversified, focusing on altering chain length, creating points of unsaturation along the chain, and including an aryl or

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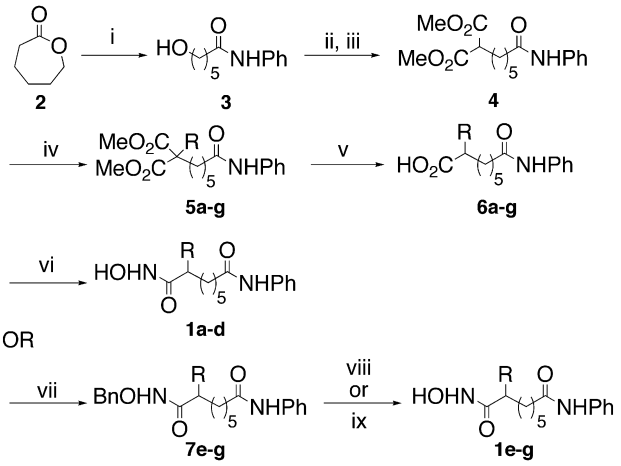
cyclohexyl ring within the chain.[19–22] However, few studies have examined the impact of substituents on the linker chain. Recently, small molecules bearing substituents on the linker adjacent to the capping group were shown to not only display nanomolar inhibition, but also modest isoform selectivity.[15] In contrast, the incorporation of substituents on the linker adjacent to the metal binding moiety has a variable influence on inhibitory activity. Two reports noted that a methyl substituent near the hydroxamic acid of hydroxamate based libraries led to 120- to 170-fold decreased HDAC inhibition compared to the unsubstituted analog, although the potency was in the micromolar range.[23,24] In addition, the short chain fatty acid valproic acid contains an propyl group adjacent to the carboxylate and inhibits in the micromolar range. In contrast, small molecules bearing an intra-chain aryl group near the hydroxamic acids display nanomolar HDAC inhibition.[20,21] For example, MS-275 bears an aryl group adjacent to the benzamide group and displays potent HDAC inhibition.[11] Therefore, the influence of substituents on the linker adjacent to the hydroxamic acid remains unclear. The structural requirements of HDAC inhibitors, particularly on the linker chain, are an interesting and relatively unexplored area of study. Modification of known HDAC inhibitors is necessary to identify the structural factors influencing inhibitor potency and provide insight for designing new inhibitors.

To probe the structural requirements of HDAC inhibitors, analogs of SAHA with substituents adjacent to the hydroxamic acid were tested. Specifically, we synthesized a small library of SAHA analogues (**1**) bearing a variety of hydrophobic substituents at the C2 position.[25] Hydrophobic substituents were selected because crystallographic analysis of HDAC proteins indicates that the active site residues surrounding the linker chain are hydrophobic.[4–6] The synthetic route for the small molecule library is outlined in Scheme 1. ε-Caprolactone (**2**) was opened with aniline to give anilide alcohol **3**. The alcohol was activated prior to incubation with the anion of dimethyl malonate to give diester **4**. The diester was deprotonated and treated with a variety of alkyl halides to afford compounds **5a**–**g**. Krapcho type decarboxylation[26] and subsequent saponification gave compounds **6a**–**g**. Carboxylic acids **6a**–**d** were converted directly to the hydroxamic acids **1a**–**d** with ethyl chloroformate and a hydroxylamine solution. However, the low yields encouraged utilization of a second strategy where hydroxamic acids **1e**–**g** were synthesized via the benzyl protected hydroxamic acids **7e**–**g** followed by deprotection by either H_2/Pd–C for compound **1g**, or BCl_3[27] for unsaturated compounds **1e** and **1f**. Yields after the two-step hydroxamic acid installation/benzyl deprotection were superior to direct conversion (38% and 64% compared to 10–24%). Although reported for benzyl ethers, we note that use of BCl_3 to remove a benzyl group on a hydroxamic acid is unestablished to the best of our knowledge.[28] A more thorough exploration of the scope and limitations of the BCl_3 deprotection reaction is currently under investigation.

HDAC inhibitory activities of the SAHA analogs were measured using the Fluor de Lyse™ in vitro fluorescence activity assay kit.[29] IC_{50} values were obtained by fitting the data to a sigmoidal dose–response curve (Fig. 2). The structure–activity relationship of compounds **1a**–**g** is summarized in Table 1. All of the SAHA analogs inhibited HDAC activity in the micromolar range. The butyl variant **1d** was the most potent analog displaying an IC_{50} of 72 μM, while the ethyl variant **1b** displayed the weakest inhibitory activity of 449 μM. Interestingly, the analogs containing the smallest (**1a**-methyl) or the largest (**1g**-benzyl) substituents displayed HDAC inhibitory activities between those of **1d** and **1b**. In addition, the propargyl analog **1f** inhibited to almost the same level as the butyl analog **1d** (87 and 72 μM, respectively). The results indicate steric considerations alone cannot predict the inhibitory activities of the C2-substituted analogs.



Scheme 1. Reagents and conditions for the racemic synthesis of compounds **1a**–**g**: (i) $PhNH_2$, $AlMe_3$, THF, 98%; (ii) MsCl, TEA, CH_2Cl_2, 99%; (iii) a—NaH, dimethylmalonate, THF; b—mesylate from (ii), THF, reflux, 90%; (iv) NaH, RX, THF, reflux, 33–98%; (v) a—LiCl, H_2O, DMSO, reflux; b—NaOH, MeOH, reflux, 67–83%; (vi) Ethyl chloroformate, *N*-methylmorpholine, NH_2OH, MeOH, 10–24%; (vii) CDI, TEA, NH_2OBn, THF, reflux, 75–91%; (viii) H_2, Pd–C, MeOH, 48%; (ix) BCl_3, CH_2Cl_2, 84–85%.

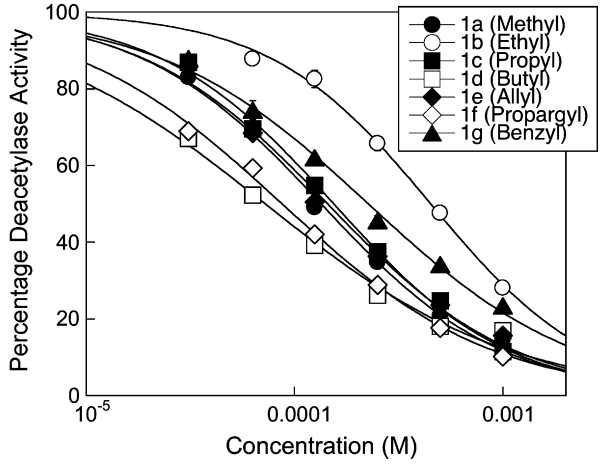


Figure 2. Dose–response curves of SAHA analogues **1a**–**g** from three independent trials with error bars indicating standard error.

Table 1. HDAC inhibition by compounds **1a**–**g**, SAHA, and MS-275

Compounds	R	IC_{50}[a] (μM)
SAHA		0.090 (±0.004)
MS-275		3.2 (±0.1)
1a	Methyl	134 (±6)
1b	Ethyl	449 (±17)
1c	*n*-Propyl	154 (±7)
1d	*n*-Butyl	72 (±6)
1e	Allyl	144 (±9)
1f	Propargyl	87 (±5)
1g	Benzyl	226 (±11)

[a] Values are means of three experiments with standard error given in parentheses.

While the SAHA analogs displayed inhibitory activities in the micromolar range, all had significantly decreased inhibitory activities when compared to those of SAHA (90 nM) or MS-275 (3.2 μM). The most potent butyl variant **1d** demonstrated 800- and 20-fold decreased activity compared to SAHA and MS-275, respectively. The weakest ethyl variant **1b** displayed 5000- and 128-fold decreased inhibition compared to SAHA and MS-275. The results suggest that any group, regardless of size, incorporated adjacent to the hydroxamic acid will result in decreased inhibitory activity compared to the unsubstituted analog.

Several HDAC inhibitors maintain similar nanomolar potency compared to SAHA yet contain a ring within the carbon linker adjacent to the metal binding moiety.[20,21] The fact that SAHA analogs with substituents at the C2 position display micromolar IC_{50} values indicates that only modest steric bulk near the hydroxamic acid is tolerated for nanomolar inhibitory activity. Therefore, the results suggest that the steric environment near the hydroxamic acid in the HDAC active site is significantly confined. The results have implications for anti-cancer drug design, predicting that HDAC inhibitors with substituents near the hydroxamic acid will have inhibitory activities in the micromolar range.

SAHA analogs with substituents adjacent to the capping group were potent nanomolar inhibitors.[15] In contrast, SAHA analogs with substituents adjacent to the hydroxamic acid demonstrated micromolar IC_{50} values. The combined data suggest that substituents are tolerated along the linker chain but potency diminishes when positioned near the metal binding moiety. Because modest isoform selectivity has been reported with HDAC inhibitors bearing substituents along the linker,[15,17] a systematic assessment of substituent tolerance along the linker chain will guide future HDACi design. The effect of incorporating substituents at additional positions along the linker chain is currently under investigation.

Acknowledgments

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25. Hydroxamic acid characterization. No spurious peaks were observed in the NMR spectra of the synthesized compounds. Compound **1a**: 33% yield from **6a**. Mp 128–130 °C; ^{1}H NMR (500 MHz, CD_3OD) δ (ppm): 1.3 (d, 3H), 1.35–1.4 (m, 6H), 1.6–1.7 (m, 4H), 2.2 (m, 1H), 2.4 (t, 2H), 7.1 (t, 1H), 7.3 (t, 2H), 7.5 (t, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ (ppm): 17.0, 25.0, 27.0, 29.0, 34.0, 37.0, 38.0, 120.0, 124.0, 129.0, 139.0, 173.0, 175.0; HRMS (ESI-LC–MS, *m*/*z*); found: [M], 278.1640, calcd for $C_{15}H_{22}N_2O_3$, 278.1630. Compound **1b**: 14% yield from **6b**. Mp 121–123 °C; ^{1}H NMR (500 MHz, CD_3OD) δ (ppm): 0.9 (t, 3H), 1.25–1.5 (m, 6H), 1.6 (m, 2H), 1.7 (m, 2H), 1.95 (m, 1H), 2.4 (t, 2H), 7.1 (t, 1H), 7.3 (t, 2H), 7.5 (t, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ (ppm): 11.0, 25.8, 25.9, 27.1, 29.0, 32.3, 36.7, 45.0, 120.0, 124.0, 129.0, 139.0, 173.0, 175.0; HRMS (ESI-LC–MS, *m*/*z*); found: [M−H_2O], 274.1687, calcd for $C_{16}H_{22}N_2O_2$, 274.1681. Compound **1c**: 10% yield from **6c**. Mp 164–166 °C; ^{1}H NMR (500 MHz, CD_3OD) δ (ppm): 0.9 (t, 3H), 1.25–1.5 (m, 8H), 1.6 (m, 2H), 1.7 (m, 2H), 2.1 (m, 1H), 2.35 (t, 2H), 7.1 (t, 1H), 7.3 (t, 2H), 7.5 (t, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ (ppm): 13.0, 20.7, 25.9, 27.0, 29.0, 33.0, 35.0, 37.0, 43.7, 120.0, 124.0, 129.0, 139.0, 173.5, 175.0; HRMS (ESI-LC–MS, *m*/*z*); found: [M−H_2O], 288.1851, calcd for $C_{17}H_{24}N_2O_2$, 288.1838. Compound **1d**: 24% yield from **6d**. Mp 153–156 °C; ^{1}H NMR (500 MHz, CD3OD) δ (ppm): 0.9 (t, 3H), 1.2–1.5 (m, 10H), 1.6 (m, 2H), 1.7 (m, 2H), 2.05 (m, 1H), 2.35 (t, 2H), 7.1 (t, 1H), 7.3 (t, 2H), 7.5 (t, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ (ppm): 13.0, 22.5, 25.5, 27.0, 29.0, 30.0, 32.4, 32.5, 37.0, 44.0, 120.0, 124.0, 129.0, 139.0, 173.0, 175.0; HRMS (ESI-LC–MS, *m*/*z*); found: [M−H_2O], 302.2000, calcd for $C_{18}H_{26}N_2O_2$, 302.1994. Compound **1e**: 84% yield from **7e**. Mp 143–146 °C; ^{1}H NMR (500 MHz, CD_3OD) δ (ppm): 1.25–1.5 (m, 4H), 1.6 (m, 2H), 1.7 (m, 2H), 2.1 (m, 2H), 2.3 (m, 1H), 2.35 (t, 2H), 5.0 (q, 2H), 5.7 (m, 1H), 7.1 (t, 1H), 7.3 (t, 2H), 7.5 (t, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ (ppm): 25.5, 27.0, 29.0, 32.0, 36.5, 37.0, 44.0, 116.0, 120.05, 124.0, 129.0, 136.0, 139.0, 174.0; HRMS (ESI-LC–MS, *m*/*z*); found: [M], 304.1797, calcd for $C_{17}H_{24}N_2O_3$, 304.1787. Compound **1f**: 85% yield from **7f**. Mp 138–140 °C; ^{1}H NMR(500 MHz, CD_3OD) δ (ppm): 1.25–1.5 (m, 4H), 1.6 (m, 2H), 1.7 (m, 2H), 2.2–2.45 (m, 6H), 7.1 (t, 1H), 7.3 (t, 2H), 7.5 (t, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ (ppm): 22.0, 25.0, 27.0, 29.0, 32.0, 37.0, 43.5, 70.3, 81.3, 120.0, 124.0, 129.0, 139.0, 173.0, 175.0; HRMS (ESI-LC–MS, *m*/*z*); found: [M], 302.1640, calcd for $C_{17}H_{22}N_2O_3$, 302.1630. Compound **1g**: 48% yield from **7g**. Mp 138–140 °C; ^{1}H NMR (500 MHz, CD_3OD) δ (ppm): 1.3 (m, 4H), 1.5 (m, 2H), 1.7 (m, 2H), 2.35 (m, 2H), 2.65 (m, 1H), 2.7 (m, 1H), 2.9 (m, 1H), 7.1 (t, 1H), 7.17 (m, 3H), 7.2 (t, 2H), 7.3 (t, 2H), 7.5 (t, 2H); ^{13}C NMR (125 MHz, CD_3OD) δ (ppm): 25.0, 27.0, 29.0, 37.0, 39.0, 46.0, 120.0, 124.0, 126.0, 128.0, 128.5, 129.0, 139.0, 140.0, 173.0, 174.0; HRMS (ESI-LC–MS, *m*/*z*); found: [M], 354.1957, calcd for $C_{21}H_{26}N_2O_3$, 354.1943.
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27. In a representative procedure, 0.12 g (0.3 mmol) of *O*-benzyl protected hydroxamic acid **7e** was dissolved in 3 ml THF and cooled to 0 °C via an ice bath. 1.5 ml (5 equiv) of a 1 M BCl_3 solution in CH_2Cl_2 was then added dropwise. The ice bath was removed after 5 min and the reaction mixture was stirred for an additional 3 h (complete by TLC analysis) at room temperature. The mixture was then quenched with 1 M HCl and extracted with ethyl acetate (3× 10 ml). The organic layers were pooled, dried over magnesium sulfate, and evaporated onto silica gel. Flash chromatography (10% MeOH/CH_2Cl_2) with acid-washed (deferrated) silica gel afforded the hydroxamic acid **1e** in 84–85% yield. For a representative procedure for obtaining deferrated silica gel, see Guo, H.; Naser, S. A.; Ghobrial, G.; Phanstiel, O. *J. Med. Chem.* **2002**, *45*, 2056.
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29. The HDAC activity was measured using Fluor de Lyse™ activity assay (Biomol). Briefly, HeLa lysates (25 μL) were incubated with or without the small molecule inhibitor for 30 min at 30 °C with shaking. Fluor de Lys substrate (25 μL, 100 μM) was added and the reaction mixture was incubated at 37 °C for 45 min with shaking. Fluor de Lys developer (50 μL of 1×) was added and incubated with shaking for 5 min. The fluorescence intensity was determined at 465 nm using a Genios Fluorimeter (Tecan). The deacetylase activity was determined by dividing the fluorescence intensity of the reaction in the presence of SAHA analog with the intensity in the absence of inhibitor. At least three determinations were used to calculate the mean and standard error in Figure 2 and Table 1.