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Jessica L. Tischler

Basel Abuaita

Sierra C. Cuthpert

Christopher Fage

Kristi Murphy

See next page for additional authors

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Authors

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Simple inhibitors of histone deacetylase activity that combine features of short-chain fatty acid and hydroxamic acid inhibitors

JESSICA L. TISCHLER¹, BASEL ABUAITA¹, SIERRA C. CUTHPERT¹, CHRISTOPHER FAGE¹, KRISTI MURPHY¹, ANDREW SAXE², EDWARD B. FURR³, JAMIE HEDRICK³, JENNIFER MEYERS³, DAVID SNARE³, & ALI R. ZAND³

¹Department of Chemistry and Biochemistry, University of Michigan-Flint, Flint, MI, USA, ²McLaren Regional Medical Center, Flint, MI, USA, and ³Department of Science and Mathematics, Kettering University, Flint, MI, USA

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Abstract

Butyric acid and trichostatin A (TSA) are anti-cancer compounds that cause the upregulation of genes involved in differentiation and cell cycle regulation by inhibiting histone deacetylase (HDAC) activity. In this study we have synthesized and evaluated compounds that combine the bioavailability of short-chain fatty acids, like butyric acid, with the bidentate binding ability of TSA. A series of analogs were made to examine the effects of chain length, simple aromatic cap groups, and substituted hydroxamates on the compounds' ability to inhibit rat-liver HDAC using a fluorometric assay. In keeping with previous structure-activity relationships, the most effective inhibitors consisted of longer chains and hydroxamic acid groups. It was found that 5-phenylvaleric hydroxamic acid and 4-benzoylbutyric hydroxamic acid were the most potent inhibitors with IC₅₀'s of 5 μM and 133 μM respectively.

Keywords: *Histone deacetylase, short-chain fatty acid, hydroxamic acid, inhibitor, cancer*

Introduction

Reversible modification of chromatin's structure plays a fundamental role in regulating gene transcription. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are enzymes that work in tandem to acetylate and deacetylate the ε-amino groups on the lysine residues located in the N-terminal region of the core histone proteins [1–3]. Acetylation of these residues by HATs neutralizes the histones' positive charge and decreases the DNA's affinity for the protein. The DNA then unwinds and allows the cell's transcriptional machinery access to the strand thereby promoting gene expression. HDACs reverse this process by cleaving the acetyl groups. This results in a closed chromatin structure and the repression of gene expression. Inhibition of HDAC activity

therefore has the effect of promoting the transcription of silenced genes. Application of HDAC inhibitors in cancer cell lines and mouse model systems leads to interruption of the cell cycle, differentiation, and apoptosis [3–6]. Clinical studies have shown tumor repression and improvement of patient symptoms without significant side effects. These therapeutic effects are a result of increased expression of proteins such as p21^{WAF1/CIP1}, FAS, and caspase 3 [7–9].

Structure-activity relationship studies of various HDAC enzymes and X-ray crystal structures of inhibitors bound to a histone deacetylase-like protein (HDLP) from *Aquifex aeolicus* have elucidated HDACs' conserved active site [10–12]. Hydrolysis of the acetylated lysine residue is promoted by coordination of the carbonyl oxygen with a Zn⁺²

Correspondence: J.L. Tischler, Department of Chemistry and Biochemistry, University of Michigan-Flint, 303 E. Kearsley St., Flint, MI 48502, USA. Fax: 810-766-6693. E-mail: jtisch@umflint.edu. A.R. Zand, Department of Science and Mathematics, Kettering University, 1700 W. Third Ave., Flint, MI 48504, USA. Fax: 810-762-7979. E-mail: azand@kettering.edu

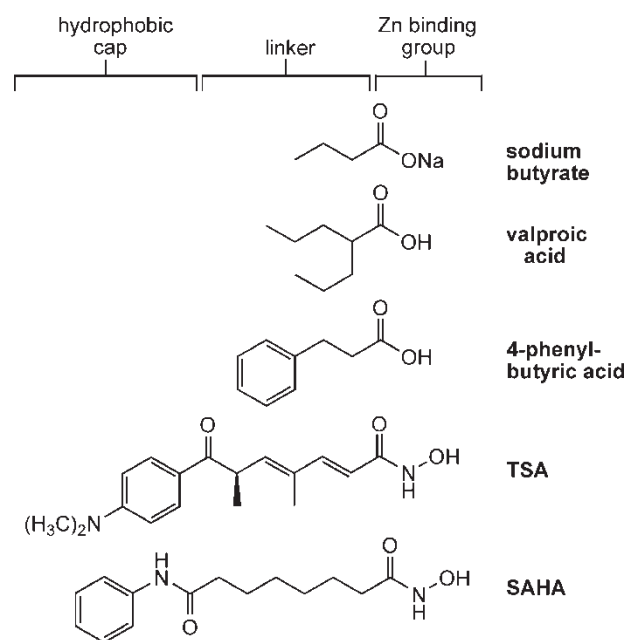


Figure 1. Structures of known HDAC inhibitors.

cation. The Zn^{+2} cation lies at the bottom of a hydrophobic channel, greatly influencing the structure of inhibitors. Although many classes of inhibitors have been developed, they all have a Zn-binding moiety located at the end of hydrophobic linker chain for proper placement in this channel (Figure 1). Opposite the Zn-binding group, most inhibitors incorporate a hydrophobic cap structure that has been shown to interact with aromatic residues at the mouth of the channel. Consequently, many of these groups are aromatic in nature, but can vary greatly in size and secondary functionality.

One of the first compounds found to inhibit HDAC was sodium butyrate (Figure 1). It is representative of the short-chain fatty acid class of inhibitors. A fatty acid found naturally in the body, its biological effects have been well studied [13,14]. Although a non-specific, millimolar level inhibitor of HDAC, it has been found to induce differentiation in neuroblastoma cells, human ovarian carcinoma cells, and leukemia cells, among others [13]. However, rapid metabolism has limited its clinical development due to the need for constant infusion to maintain bioactive levels [15]. Investigations of other short chain acids, such as 4-phenylbutyric acid and valproic acid (Figure 1), have shown them to be effective against similar cancer cell lines [16,17]. Although 4-phenylbutyric acid's *in vitro* efficacy is low, it was the first HDAC inhibitor to be evaluated in clinical trials [3]. Valproic acid, a drug currently used to treat epilepsy and bipolar disorder, has a significantly longer half-life than sodium butyrate and has an established clinical record [16]. Depending on the type of HDAC studied, IC_{50} values for valproic acid have been measured between

0.5 – 2 mM [18,19]. Despite its low potency, valproic acid was studied as a combination therapy with retinoic acid in patients with acute myloid leukemia [20].

Originally isolated from *Streptomyces hygroscopicus* as an antifungal antibiotic agent, (*R*)-Trichostatin A (TSA) (Figure 1) was found to induce cell differentiation [4,21]. Further studies revealed HDACs to be its molecular target. TSA, with its ability to bind Zn^{+2} in a bidentate fashion, not only is one of the most potent inhibitors of HDAC with an IC_{50} less than 10 nM, but it also spawned the investigation into the largest class of HDAC inhibitors — the hydroxamic acids. Studies with hydroxamic acids and longer chain fatty acids led to the development of suberoylanilide hydroxamic acid (SAHA) (Figure 1), which is currently in phase III clinical trials [3,22]. Crystallographic studies of TSA and SAHA bound to HDLP show that the hydroxamic acid not only provides additional binding to the Zn^{+2} via the N-hydroxyl group, but the nitrogen and hydroxyl species also serve as H-bond donors to active-site histidines. Although SAHA is expected to be introduced on the market in the near future [23], TSA has had limited clinical success. TSA is now commercially available in enantiopure form, but the costly asymmetric synthesis hampers its pharmaceutical development. An earlier study with mouse xenographs also showed no antitumor activity. It was suggested that this could be due in part to TSA's poor solubility [24]. There is also mixed data regarding the toxicity of hydroxamic acids in general. Multiple animal and clinical studies on TSA and SAHA have not shown general toxicity or teratogenicity [25]. However, earlier studies of hydroxamic acid metabolism show that hydrolysis can produce hydroxylamine, which is a potential mutagen [26]. Pharmacokinetic studies of TSA have shown that hydroxamic acids are prone to glucuronidation, sulfonation, and reduction to the amide [26]. Enolization to the *S*-enantiomer or hydrolysis to the corresponding carboxylic acid also results in inactivity. This metabolic instability results in a relatively short half-life *in vivo* [28].

The goals of this study are to combine the beneficial structural features of both short-chain fatty acids and hydroxamic acids. Short-chain acids have improved solubility, low toxicity, and are relatively easy to obtain and derivitize. Hydroxamates provide increased inhibition via bidentate binding to the Zn^{+2} and additional hydrogen bonds. 5-Phenylvaleric acid, 3-benzoylpropanoic acid, 3-benzoylbenzoic acid, and 4-benzoylbenzoic acid were selected as synthetic starting points not only for their similarity to 4-phenylbutyric acid, but because they are relatively inexpensive and commercially available. 4-Phenylbutyric acid derivatives were also developed for comparison. The hydroxamic acid, N-methyl hydroxamate and N,O-dimethyl hydroxamate derivatives of

these compounds were synthesized and their inhibition of rat-liver HDAC was investigated. The methylated hydroxamates were developed as potential alternatives to hydroxamic acids. It is hypothesized that these compounds will still bind to the Zn^{+2} in a bidentate fashion while potentially reducing the metabolic concerns. Because the methyl groups could lead to negative steric interactions and eliminate key hydrogen bonds, the inhibition of these compounds was compared to the corresponding carboxylic and hydroxamic acids in this study.

Materials and methods

General

All of the carboxylic acid starting materials were purchased from Aldrich (Milwaukee, WI) and were used without further purification. 1H and ^{13}C NMR spectra were obtained using a 300 MHz Varian Gemini instrument. FTIR spectra were obtained using either a Thermo Nicolet Avatar 360 FT-IR ESP or a MIDAC M series FTIR. Elemental analysis was performed using a Perkin-Elmer 2400 series II CHN analyzer. Mass spectroscopy data was obtained using a Micromass LCT Time-of-Flight Mass Spectrometer with Electrospray and APCI. Elemental analysis and mass spectroscopy studies were performed by the Department of Chemistry's facilities at the University of Michigan in Ann Arbor, MI.

General procedure for synthesis of hydroxamates

The desired carboxylic acid (0.1 moles) was added to 150 mL of dry THF in a nitrogen flushed flask. N-methylmorpholine (0.1 moles) was added and the solution/mixture was stirred at room temperature under nitrogen atmosphere for 0.5 h. Isopropyl chloroformate (0.12 moles) was added drop-wise producing a cloudy solution. This mixture was stirred at room temperature for 2.5 h before a solution containing the desired hydroxylamine (0.12 moles) and N-methylmorpholine (0.12 moles) in dry acetonitrile was added. The resulting mixture was stirred overnight. Following removal of the solvent using rotary evaporation, the resulting slurry was dissolved in 10% HCl and extracted three times with ethyl acetate. The combined organic layers were extracted twice with saturated sodium carbonate, followed by sodium chloride, and then dried over anhydrous magnesium sulfate. The solvent was evaporated to yield a yellowish oil which was chromatographed, using hexane/ethyl acetate mixtures (gradient chromatography) as eluent, to yield white or off-white solids or oils as products (Figure 2). Yields ranged from 68% to 80%.

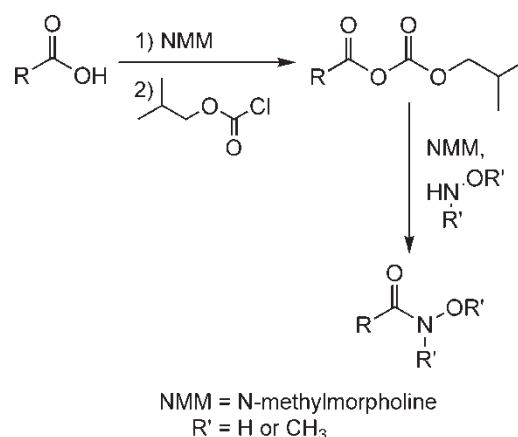


Figure 2. General synthesis of hydroxamate, N-methylhydroxamates, and N,O-dimethylhydroxamates.

4-Phenylbutyric acid hydroxamate (2). Melting point: 73–77°C. 1H NMR ($CDCl_3$): δ 1.95 (quintet, $J = 7.5$ Hz, 2H), 2.36 (t, $J = 7.5$ Hz, 2H), 2.66 (t, $J = 7.8$ Hz, 2H), 7.18 (distorted triplet, $J = 8.1$ Hz, 3H), 7.27 (distorted triplet, $J = 7.8$ Hz, 2H), 9.6 (broad singlet). ^{13}C NMR ($CDCl_3$): δ 30.4, 32.34, 35.20, 125.5, 128.01, 128.09, 141.7, 171.5. IR (KBr) cm^{-1} : 3241, 3055, 3029, 2962, 1787, 1710, 1454, 1266, 1107. EA: Found: 66.92% C, 7.31% H, 7.81% N. Calculated: 67.02%, 7.31% H, 7.82% N.

4-Phenylbutyric acid N-methylhydroxamate (3). 1H NMR ($CDCl_3$): δ 2.0 (quintet, $J = 7.2$ Hz, 2H), 2.42 (t, $J = 7.5$ Hz, 2H), 2.68 (t, $J = 7.8$ Hz, 2H), 3.14 (singlet, 3H), 7.15 (distorted triplet, $J = 8.1$ Hz, 3H), 7.29 (distorted triplet, $J = 7.8$ Hz, 2H), 9.5 (broad singlet). ^{13}C NMR ($CDCl_3$): δ 30.84, 31.6, 35.42, 35.77, 125.44, 128.04, 128.1, 141.9, 174.7. IR (neat) cm^{-1} : 3175, 3031, 2939, 2864, 1618, 1490, 1201. EA: Found: 67.90% C, 7.92% H, 6.71% N. Calculated: 68.37% C, 7.32% H, 7.25% N.

4-Phenylbutyric acid N,O-dimethylhydroxamate (4). 1H NMR ($CDCl_3$): δ 1.97 (quintet, $J = 7.5$ Hz, 2H), 2.38 (t, $J = 7.5$ Hz, 2H), 2.63 (t, $J = 7.8$ Hz, 2H), 3.1 (singlet, 3H), 3.6 (singlet, 3H), 7.17 (distorted triplet, $J = 8.1$ Hz, 3H), 7.27 (distorted triplet, $J = 7.8$ Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 30.85, 32.26, 35.34, 35.86, 60.52, 125.48, 128.04, 128.1, 141.7, 174.6. IR (neat) cm^{-1} : 2938, 2868, 1672, 1497, 1387, 1094. MS: $[M + Na]^+ = 230.1151$. Calculated: 230.1157.

5-Phenylvaleric acid hydroxamate (6). Melting point: 49–53°C. 1H NMR ($CDCl_3$): δ 1.66 (quintet, $J = 3$ Hz, 4H), 2.48 (t, $J = 6$ Hz, 2H), 2.59 (t, $J = 3$ Hz, 2H), 4.2 (broad), 7.2 (distorted triplet,

$J = 6.6$ Hz, 3H), 7.31 (distorted triplet, $J = 6.6$ Hz, 2H), 9.5 (broad singlet). ^{13}C NMR (CDCl_3): δ 24.72, 30.41, 32.3, 35.15, 125.48, 128.0, 128.1, 141.72, 171.4. IR (KBr) cm^{-1} : 3202, 3030, 2928, 2861, 1618, 1458. MS: $[\text{M} + \text{H}]^+ = 294.1172$. Calculated: 294.1172.

5-Phenylvaleric acid N-methylhydroxamate (7). ^1H NMR (CDCl_3): δ 1.70 (quintet, $J = 3$ Hz, 4H), 2.58 (t, $J = 6$ Hz, 2H), 2.66 (t, $J = 3$ Hz, 2H), 3.26 (s, 3H), 7.2 (distorted triplet, $J = 6.6$ Hz, 3H), 7.29 (distorted triplet, $J = 6.6$ Hz, 2H), 9.5 (broad singlet). ^{13}C NMR (CDCl_3): δ 24.28, 30.94, 31.76, 35.4, 35.8, 125.48, 128.04, 128.1, 141.95, 174.4. IR (neat) cm^{-1} : 3190, 3023, 2939, 2871, 1611, 1497, 1459, 1383, 1201. MS: $[\text{M} + \text{H}]^+ = 208.1336$. Calculated: 208.1338.

5-Phenylvaleric acid N,O-dimethylhydroxamate (8). ^1H NMR (CDCl_3): δ 1.72 (quintet, $J = 3$ Hz, 4H), 2.50 (t, $J = 6$ Hz, 2H), 2.62 (t, $J = 3$ Hz, 2H), 3.16 (s, 3H), 3.72 (s, 3H), 7.2 (distorted triplet, $J = 6.6$ Hz, 3H), 7.29 (distorted triplet, $J = 6.6$ Hz, 2H). ^{13}C NMR (CDCl_3): δ 24.30, 30.85, 32.26, 35.34, 35.86, 60.52, 125.48, 128.04, 128.1, 141.95, 174.6. IR (neat) cm^{-1} : 3084, 3031, 2939, 2864, 1672, 1452, 1383. EA: Found: 70.55% C, 8.59% H, 5.91% N. Calculated: 70.56% C, 8.65% H, 6.33% N.

3-Benzoylpropanoic acid hydroxamate (10). Melting point: 96–98°C. ^1H NMR (CDCl_3): δ 2.92 (t, $J = 6.6$ Hz, 2H), 3.37 (t, $J = 6.6$ Hz, 2H), 7.45 (tt, $J = 7.2$ Hz, $J = 1.2$ Hz, 2H), 7.53 (tt, $J = 7.5$ Hz, $J = 1.2$ Hz, 1H), 8.0 (dt, $J = 6.9$ Hz, $J = 1.8$ Hz, 2H), 9.6 (broad singlet, 1H). ^{13}C NMR (CDCl_3): δ 25.6, 31.4, 127.7, 128.33, 132.7, 136.3, 172.9, 198.3. IR (KBr) cm^{-1} : 3405, 3055, 2987, 1686, 1627, 1449, 1422, 1267. EA: Found: 62.14% C, 5.76% H, 7.24% N. Calculated: 62.17% C, 5.74% H, 7.25% N.

3-Benzoylpropanoic acid N-methylhydroxamate (11). Melting point: 103–105°C. ^1H NMR (CDCl_3): δ 2.85 (t, $J = 6.6$ Hz, 2H), 3.2 (s, 3H), 3.28 (t, $J = 6.6$ Hz, 2H), 7.43 (tt, $J = 7.2$ Hz, $J = 1.2$ Hz, 2H), 7.6 (tt, $J = 7.5$ Hz, $J = 1.2$ Hz, 1H), 8.02 (dt, $J = 6.9$ Hz, $J = 1.8$ Hz, 2H), 9.44 (broad singlet, 1H). ^{13}C NMR (CDCl_3): δ 25.56, 31.8, 32.52, 127.59, 128.3, 132.66, 136.3, 172.8, 198.7. IR (KBr) cm^{-1} : 3144, 2944, 2876, 2718, 1674, 1603, 1508, 1201. EA: Found: 63.65% C, 6.56% H, 6.83% N. Calculated: 63.76% C, 6.32% H, 6.76% N.

3-Benzoylpropanoic acid N,O-dimethylhydroxamate (12). ^1H NMR (CDCl_3): δ 2.87 (t, $J = 6.6$ Hz, 2H), 3.18 (s, 3H), 3.32 (t, $J = 6.6$ Hz, 2H), 3.753 (s, 3H), 7.43 (tt, $J = 7.2$ Hz, $J = 1.2$ Hz, 2H), 7.53 (tt, $J = 7.5$ Hz, $J = 1.2$ Hz, 1H), 7.98 (dt, $J = 6.9$ Hz, $J = 1.8$ Hz, 2H). ^{13}C NMR (CDCl_3): δ 25.62, 31.78, 32.52, 60.79, 127.63, 128.13, 132.73, 136.33, 172.85, 198.49. IR (neat) cm^{-1} : 3065, 2669, 2940, 2917, 1682, 1661, 1449, 1362, 1242, 1181, 1001. EA: Found: 65.24% C, 6.96% H, 5.39% N. Calculated: 65.14% C, 6.83% H, 6.33% N.

3-Benzoylbenzoic acid hydroxamate (14). Melting point: 147–148°C. ^1H NMR (CDCl_3): δ 7.35 (m, 3H), 7.49 (t, $J = 7.5$ Hz), 7.69 (m, 4H), 7.94 (broad singlet, 1H), 9.50 (broad, 1H). ^{13}C NMR (CDCl_3): δ 128.2, 129.4, 129.82, 131.62, 132.38, 136.45, 137.7, 171.4, 196.1. IR (KBr) cm^{-1} : 3318, 3059, 2816, 1653, 1622, 1561, 1285. EA: Found: 69.90% C, 4.79% H, 5.87% N. Calculated: 69.70% C, 4.60% H, 5.81% N.

3-Benzoylbenzoic acid N-methylhydroxamate (15). Melting point: 81–83°C. ^1H NMR (CDCl_3): δ 3.25 (s, 3H), 7.40 (m, 3H), 7.53 (t, $J = 7.5$ Hz), 7.72 (m, 4H), 7.9 (broad singlet, 1H), 9.34 (broad, 1H, OH). ^{13}C NMR (CDCl_3): δ 36.9, 128.3, 129.32, 129.76, 131.72, 132.6, 136.4, 137.7, 172.1, 195.5. IR (KBr) cm^{-1} : 3117, 3059, 2816, 1667, 1613, 1578, 1323, 1275, 1215. EA: Found: 70.56% C, 5.47% H, 5.42% N. Calculated: 70.58% C, 5.13% H, 5.49% N.

3-Benzoylbenzoic acid N,O-dimethylhydroxamate (16). ^1H NMR (CDCl_3): δ 3.25 (s, 3H), 3.77 (s, 3H), 7.37 (m, 3H), 7.50 (t, $J = 7.5$ Hz), 7.67 (m, 4H), 7.90 (broad singlet, 1H). ^{13}C NMR (CDCl_3): δ 37.8, 61.14, 128.12, 129.27, 129.7, 131.66, 132.58, 136.42, 137.7, 171.09, 195.8, 171.09, 195.8. IR (neat) cm^{-1} : 3067, 2938, 1780, 1723, 1661, 1447, 1279, 1172. MS: $[\text{M} + \text{Na}]^+ = 292.0948$. Calculated: 292.0950.

4-Benzoylbutyric acid hydroxamate (18). Melting point: 115–118°C. ^1H NMR (CDCl_3): δ 2.0 (quintet, $J = 6.9$ Hz, 2H), 2.42 (broad triplet, $J = 6.9$ Hz, 2H), 2.97 (t, $J = 7.2$ Hz, 2H), 7.37 (t, $J = 7.2$ Hz, 2H), 7.47 (t, $J = 7.5$ Hz, 1H), 7.90 (d, $J = 7.5$ Hz, 2H). ^{13}C NMR (CDCl_3): δ 18.65, 30.42, 31.58, 127.8, 128.2, 132.63, 136.50, 173.62, 199.7. IR (KBr) cm^{-1} : 3177, 3065, 3027, 2926, 1744, 1707, 1611, 1453. EA: Measured: 63.42% C, 6.43% H, 9.19% N. Calculated: 63.76% C, 6.32% H, 6.76% N.

4-Benzoylbutyric acid N-methylhydroxamate (19). Melting point: 89–91°C. ¹H NMR (CDCl₃): δ 2.08 (quintet, J = 6.9 Hz, 2H), 2.45 (broad triplet, J = 6.9 Hz, 2H), 3.05 (t, J = 7.2 Hz, 2H), 3.17 (s, 3H), 7.37 (t, J = 7.2 Hz, 2H), 7.47 (t, J = 7.5 Hz, 1H), 7.90 (d, J = 7.5 Hz, 2H). ¹³C NMR (CDCl₃): δ 18.42, 30.60, 31.6, 37.2, 127.7, 128.31, 132.46, 136.7, 173.5, 199.2. IR (KBr) cm⁻¹: 3175, 2897, 1672, 1611, 1447, 1181. EA: Found: 65.30% C, 7.19% H, 6.22% N. Calculated: 65.14% C, 6.83% H, 6.33% N.

4-Benzoylbutyric acid N,O-dimethylhydroxamate (20). ¹H NMR (CDCl₃): δ 2.01 (quintet, J = 6.9 Hz, 2H), 2.48 (broad triplet, J = 6.9 Hz, 2H), 3.01 (t, J = 7.2 Hz, 2H), 3.10 (s, 3H), 3.59 (s, 3H), 7.37 (t, J = 7.2 Hz, 2H), 7.47 (t, J = 7.5 Hz, 1H), 7.90 (d, J = 7.5 Hz, 2H). ¹³C NMR (CDCl₃): δ 18.71, 30.52, 31.78, 37.35, 60.8, 127.7, 128.2, 132.6, 136.47, 173.7, 199.5. IR (neat) cm⁻¹: 3061, 2940, 1686, 1449, 1231, 1185, 993. MS: [M + Na]⁺ = 258.1112. Calculated: 258.1106.

HDAC assay

Assay of HDAC activity was modified from the method of Wegener [29]. The assay is a two-step reaction. During the first step, HDAC deacetylates the ε-acetylated lysine residue of the substrate. In the second step, 4-amino-7-methylcoumarin is cleaved from the deacetylated substrate using trypsin, causing a significant increase in fluorescence compared to the acetylated substrate. Assays were run in triplicate with positive and negative controls in at least three separate experiments.

The peptide substrate of AC-Arg-Gly-Lys(AC)-AMC (Bachem, USA; 5 mg) was initially dissolved in DMSO and diluted in HDAC buffer (15 mM Tris-HCl [pH 8.1], 250 μM EDTA, 250 mM NaCl, 10% glycerol) to give a 1 mM solution. Rat liver HDAC (Calbiochem, Germany; 89 U/mL) was diluted in HDAC buffer. Stock solutions of all inhibitors were made in DMSO.

Enzyme reactions were prepared by combining HDAC buffer, the diluted enzyme (3 U/mL), and 10 μL of inhibitor stock solutions to achieve a final concentration of 10% DMSO. Following 5 min of incubation at 30°C, the reaction was initiated by the addition of substrate (300 μM) to bring the total volume to 100 μL. Following a second incubation at 30°C for 1 h, 100 μL of stop solution was added (10 mg/mL trypsin in 50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 2 μM TSA). After incubation for an additional 30 min at 30°C, the amount of free AMC was measured by monitoring the fluorescence intensity (EX = 380 nm, EM = 485 nm) using a Jobin Yvon FluoroMax-3 spectrofluorometer.

Results and discussion

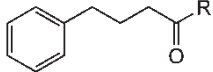
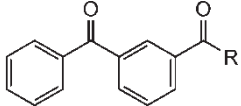
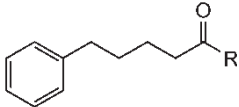
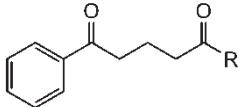
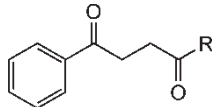
The fatty acids and the synthesized hydroxamate analogs were tested for their ability to inhibit the activity of rat-liver HDAC using a fluorescently labeled, acetylated, tripeptide mimic of the native acetylated lysine substrate. As 4-phenylbutyric acid and 5-phenylvaleric acid are reported to be millimolar-level inhibitors, all compounds were initially tested at 1 mM. The percent inhibition of these compounds is reported relative to the activity of HDAC in the presence of DMSO only (Table I). 4-Phenylbutyric acid and 5-phenylvaleric acid are consistent with the IC₅₀ values of 0.64 mM and 1.4 mM previously reported [30]. The percent inhibition of TSA at 200 nM was also measured and found to be 70–85% under these conditions. Previous studies report the IC₅₀ of TSA to be between 2–26 nM [11]. These small discrepancies can be explained due to differences in the assay method and enzyme source.

Comparison of the carboxylic acid compounds **1**, **5**, **9**, **13**, and **17** to the hydroxamic acids **2**, **6**, **10**, **14**, and **18** shows significant improvement in inhibition as would be expected by the bidentate binding of the hydroxamate to the Zn⁺² cation versus the monodentate binding of the carboxylic acid. The greatest inhibition was seen with compounds **6** and **18**. The IC₅₀ values were determined for **6** and **18** and were found to be 5 μM and 133 μM respectively (Figure 3). These values are in line with a previously determined IC₅₀ value of 1.5 μM for 5-benzoylvaleric acid hydroxamate [31]. Although the values show **6** and **18** to be less effective than other hydroxamates such as TSA and SAHA, they show increased efficacy when compared to other derivatives of 4-phenylbutyric acid and valproic acid [18].

Analysis of the 4-phenylbutyric analogs and the 3-benzoylpropanoic analogs shows the additional carbonyl on chains of equal length to have a small negative effect on inhibition. This affect is less significant when comparing chains one carbon longer in length as seen by comparison of the 5-phenylvaleric analogs to the 3-benzoylbenzoic and 4-benzoylbutyric analogs. It was hoped that this would help inhibition as amide cap groups, such as that found in SAHA, have proven to be beneficial. Perhaps the shorter chain lengths in these compounds when compared to SAHA alter the placement of the carbonyl and negate the benefit provided by this group.

Crystallographic and modeling studies of TSA and SAHA in the catalytic core of HDAC predict that three key H-bonds are formed between the carbonyl oxygen, the NH and the OH to a tyrosine and two histidines respectively. The only methylated hydroxamate previously reported was an O-methyl, phenylamide derivative of TSA with a five carbon linker chain. It had an IC₅₀ value greater than 10 μM [11]

Table I. Percent inhibition of short chain fatty acids and their hydroxamate analogs. All compounds were evaluated at 1 mM using rat liver HDAC.

Compound	Percent inhibition	Compound	Percent inhibition
4-phenylbutyric		3-benzoylbenzoic	
			
R = 1 = OH	13	R = 13 = OH	8
2 = NHOH	69	14 = NHOH	78
3 = NCH ₃ OH	42	15 = NCH ₃ OH	71
4 = NCH ₃ OCH ₃	64	16 = NCH ₃ OCH ₃	26
5-phenylvaleric		4-benzoylbutyric	
			
R = 5 = OH	37	R = 17 = OH	17
6 = NHOH	84	18 = NHOH	81
7 = NCH ₃ OH	39	19 = NCH ₃ OH	36
8 = NCH ₃ OCH ₃	24	20 = NCH ₃ OCH ₃	56
3-benzoylpropanoic			
			
R = 9 = OH	21		
10 = NHOH	45		
11 = NCH ₃ OH	8		
12 = NCH ₃ OCH ₃	36		

while the corresponding hydroxamate had an IC₅₀ value of 0.57 μM. Therefore, it was expected that we would see a drop in inhibition when the N-methylhydroxamate analogs (**3**, **7**, **11**, **15**, and **19**)

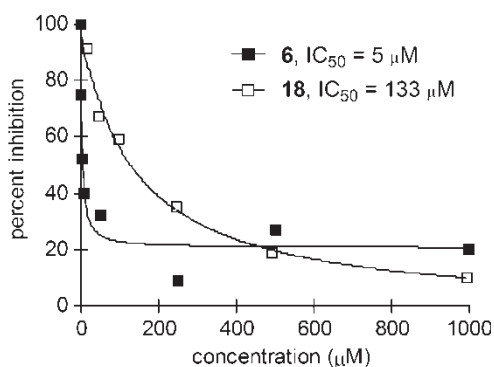


Figure 3. IC₅₀ determination of 5-phenylvaleric hydroxamic acid (**6**) and 4-benzoylbutyric hydroxamic acid (**18**).

were compared to the hydroxamates (**2**, **6**, **10**, **14**, and **18**) due to loss of one of these key H-bonds. For compounds **3**, **15**, and **19**, the N-methyl hydroxamates still provided a benefit over the carboxylic acid binding groups. A surprising result was the increased inhibition of **4**, **12**, and **20** over their N-methyl counterparts despite the loss of a second H-bond. This could be explained by increased electron donating ability of the methoxy group relative to the hydroxy resulting in tighter binding to the Zn⁺² cation. It is unclear why compounds **8** and **16** do not show this trend. They are, however, on the extremes of conformational flexibility for this series of compounds and may have increased steric or hydrophobic constraints from the two methyl groups within the active site.

The body of data collected for TSA analogs has revealed that five to six carbons in the linking region to be an optimal length for inhibition [3,11]. Hydroxamate compounds that contained a benzoyl cap group also showed an increase in inhibition with increasing chain length where six intervening methylene groups were found to be optimal [31]. Previous studies of other phenyl substituted fatty acids from 4-phenylbutyric acid through 10-phenyldecanoic acid however did not show a clear trend of increasing inhibition with increasing chain length. Our results also show increased inhibition with increased linker regions for the hydroxamic acids but not for the carboxylic acids. As the substituted hydroxamates also do not show a clear trend in this regard, it appears that when the key hydrogen bonds afforded by the hydroxamate are absent, the exact placement of the Zn binding group in the active site is less critical.

This preliminary investigation of hydroxamic acid analogs of short-chain fatty acids shows the potential of combining characteristics of these two inhibitor types. Although relatively simple in structure, phenyl or benzoyl substituted fatty acids provide an economically and synthetically accessible platform for inhibitors. Modification of the carboxylate moiety to a hydroxamate provides increased inhibition while substitution with an N,O-dimethyl hydroxamate could provide effective inhibitors with longer residence times *in vivo*.

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