

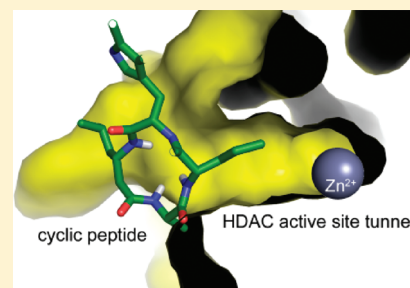
Discovery of HDAC Inhibitors That Lack an Active Site Zn²⁺-Binding Functional GroupChris J. Vickers, Christian A. Olsen,[†] Luke J. Leman, and M. Reza Ghadiri*

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Supporting Information

ABSTRACT: Natural and synthetic histone deacetylase (HDAC) inhibitors generally derive their strong binding affinity and high potency from a key functional group that binds to the Zn²⁺ ion within the enzyme active site. However, this feature is also thought to carry the potential liability of undesirable off-target interactions with other metalloenzymes. As a step toward mitigating this issue, here, we describe the design, synthesis, and structure–activity characterizations of cyclic $\alpha_3\beta$ -tetrapeptide HDAC inhibitors that lack the presumed indispensable Zn²⁺-binding group. The lead compounds (e.g., **15** and **26**) display good potency against class 1 HDACs and are active in tissue culture against various human cancer cell lines. Importantly, enzymological analysis of **26** indicates that the cyclic $\alpha_3\beta$ -tetrapeptide is a fast-on/off competitive inhibitor of HDACs 1–3 with K_i values of 49, 33, and 37 nM, respectively. Our proof of principle study supports the idea that novel classes of HDAC inhibitors, which interact at the active-site opening, but not with the active site Zn²⁺, can have potential in drug design.

KEYWORDS: HDAC inhibitors, zinc binding group, cancer therapy, tetrapeptide, apicidin



Histone deacetylases (HDACs) catalyze the removal of the acetyl group from N-Ac-lysine side chains of histone tails and other proteins.^{1,2} HDACs are among the key players in the complex epigenetic regulation of cellular processes and, consequently, are important targets for therapeutic approaches in several chronic diseases, including cystic fibrosis (CF),³ sickle cell anemia,⁴ and neurodegenerative disorders.⁵ HDAC inhibitors can also reactivate gene expression to induce cell cycle arrest and apoptosis.⁶ In that regard, there has been considerable interest in HDAC inhibitors as anticancer agents,⁷ and two drugs (**1** and **2**) have marketing approval for treating refractory cutaneous T-cell lymphoma.^{8,9} HDACs in classes 1, 2, and 4 contain a catalytic Zn²⁺ ion in the active site, which can be targeted by various compounds that possess metal binding functional groups (Figure 1). HDAC inhibitors commonly employ the following Zn²⁺-binding groups: hydroxamic acid (e.g., **1**), thiol (e.g., **2**), carboxylic acid (e.g., **3**), ketone (e.g., **4**), or 2-aminoanilide (e.g., **5**) (Figure 1).^{10,11} However, these functional groups can also bind strongly with other important metalloenzymes, leading to dose-limiting toxicity that can impede the wider application of HDAC inhibitors in pharmacotherapy.^{12,13}

We have been studying macrocyclic tetrapeptides and peptidomimetics related to apicidin (**4**) as HDAC inhibitors.^{14,15} In the course of our work, we sought to eliminate the zinc-binding group to ascertain how much residual potency could be retained. Thus, we synthesized $\alpha_3\beta$ cyclic peptide scaffolds depicted in Chart 1, based on **4**.^{14,15} The β -amino acid in the $\alpha_3\beta$ scaffold facilitates peptide cyclization during synthesis and can also impart proteolytic stability.¹⁶ All peptides

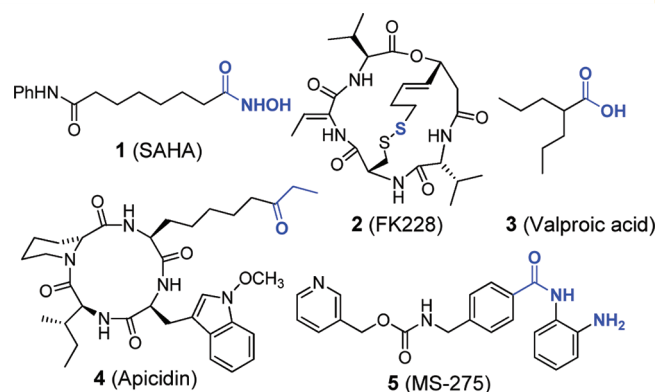


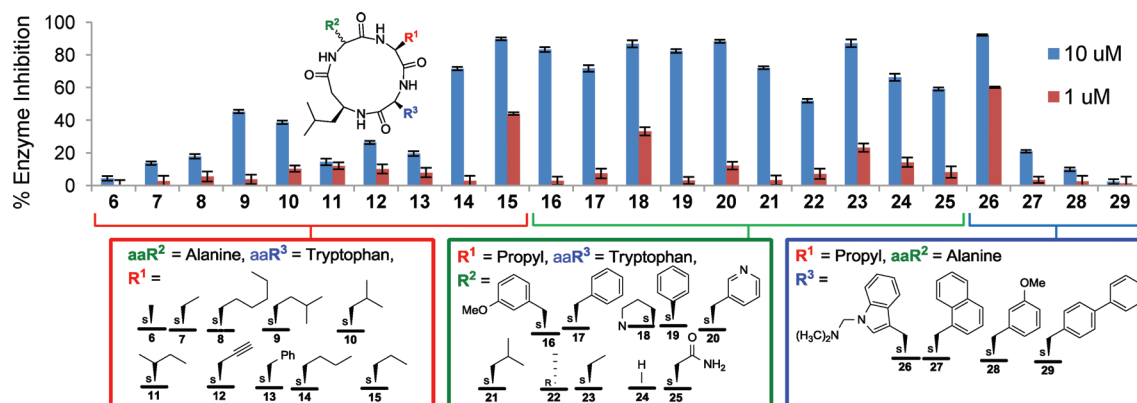
Figure 1. Common HDAC inhibitors with Zn²⁺-binding groups highlighted in blue.

were prepared using standard solid-phase peptide synthesis (SPPS) followed by cyclization in solution as described previously.^{14,15} Our initial series of compounds varied only at the R¹ position (Chart 1, red bracket, peptides **6**–**15**), which usually contains the Zn²⁺-binding group. We replaced the Zn²⁺-binding side chain with various straight-chain or branched alkyl groups of different lengths, as shown. All of the compounds were screened for inhibitory activity at concentrations of 10 and 1 μ M against recombinant HDAC3/NCOR2. Surprisingly, the

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Chart 1. SAR Data for Compounds vs HDAC3/NCoR2^a

^aPurified compounds 6–29 were synthesized and assayed against recombinant HDAC3/NCoR2 (Supporting Information). Percent inhibition data represent averages of at least two separate experiments performed in duplicate. Error bars indicate the standard deviation for each value. aaR¹, aaR², and aaR³ refer to the amino acid at position R¹, R², or R³ in the cyclic peptide.

compound containing a propyl side chain (15) was the most potent inhibitor from this series (Chart 1 and Figure S1 in the Supporting Information). It is intriguing that an alkyl chain that is shorter (6, 7) or longer (8, 14) than three carbon atoms resulted in substantially decreased activity (Chart 1 and Table S1 in the Supporting Information). We also found that incorporating unsaturation (12, 13) or branching (9–11) at position R¹ did not improve activity. Crystal structures of several Zn²⁺-dependent HDACs show a narrow binding pocket (long enough to accommodate a chain of 5–6 atoms) leading to the active-site zinc atom,¹⁷ and a recent cocrystal structure of HDAC8 bound to a cyclic depsipeptide confirmed that a long linker is necessary for binding to the zinc.¹⁸ We next prepared two additional series of compounds that varied at side chain R² (Chart 1, green bracket, compounds 16–25) or at side chain R³ (Chart 1, blue bracket, compounds 26–29) while keeping the propyl side chain at R¹ constant. The β -homoleucine amino acid was not varied, because we have found it to be optimal in HDAC inhibitors of this type.^{14,15} Compounds varied at position R² showed activity at 10 μ M; however, at 1 μ M, only the alanine and proline substitutions stood out. At position R³, where bicyclic aromatic side chain groups have been shown to facilitate inhibitor efficacy,^{14,15} 26 gave the best activity against HDAC3/NCoR2, showing 60% inhibition at 1 μ M, an unprecedented value for a molecule without a Zn²⁺-binding group (Table S1 in the Supporting Information).^{10,11} Surprisingly, the 1-naphthylalanine-containing compound (27) showed little activity even at 10 μ M, despite showing better activity than tryptophan in our previous report, where a carboxylic acid Zn²⁺-binding group was used.¹⁵

We determined IC₅₀ values for our two lead compounds, 15 and 26 (Figure 2), against a group of recombinant HDACs

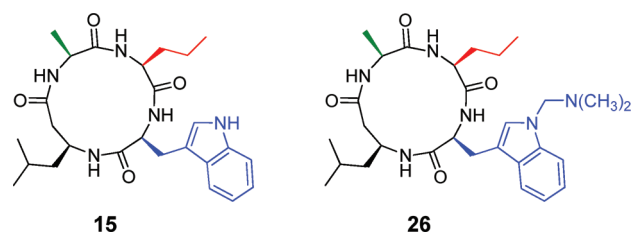


Figure 2. Lead non-Zn²⁺-binding HDAC inhibitors.

from classes 1 (HDACs 1–3) and 2b (HDAC6), which are the major deacetylase enzymes in the nucleus and cytoplasm, respectively (Table 1). Intriguingly, 26 had in vitro activity

Table 1. IC₅₀ Values for Lead HDAC Inhibitors^a

inhibitors	HDAC enzyme			
	1	2	3 ^b	6
15 ^{c,d}	0.98	1.5	1.4	>20 ^c
26 ^{c,d}	0.68	1.4	0.80	>20 ^c
SAHA ^e	0.069	0.18	0.043	0.018
FK228 ^f	0.2 nM	1.0 nM	3.0 nM	0.2
VPA ^g	39	62	161	>2000
apicidin ^h	0.022	0.035	0.029	>10
MS-275 ⁱ	0.12	0.25	0.4	>10

^aAll values are in μ M unless otherwise specified. ^bHDAC3/NCoR2. ^cCompounds 15 and 26 have 37 and 46% inhibition, respectively, against HDAC6 at 20 μ M. ^dValues are averages of at least two separate experiments performed in duplicate. ^eFrom ref 19. ^fFrom ref 20. ^gFrom ref 21. ^hFrom ref 15. ⁱFrom ref 22.

comparable to several state-of-the-art inhibitors, including being 40–200-fold more potent than valproic acid, an FDA-approved drug for epilepsy (Table 1). Compound 26 was also only 2–6-fold less active than MS-275 (Table 1), a compound in clinical trials for cancer therapy. Archetypal hydroxamic acid inhibitor 1 (SAHA) is only ~10-fold more potent against recombinant HDACs than 26 (Table 1).

Human tissue culture cytotoxicity assays were carried out to determine if our lead compounds could penetrate cells and act against intracellular HDAC complexes, which can have different properties and selectivity profiles as compared to the recombinant proteins. We screened 15, 26, apicidin (4), and SAHA (1) against several cancer cell lines, including Jurkat (T-cell leukemia), HL-60 (acute promyelocytic leukemia), HeLa (cervical cancer), MCF-7 (breast cancer), and HEK293 (human embryonic kidney) cells (Table 2). We found the best efficacy against the leukemia cell lines, where 15 caused 50% growth inhibition (GI₅₀) at 12 and 8.2 μ M against Jurkat and HL-60 cells, respectively. Compound 26 had GI₅₀ values of 8.1 and 2.8 μ M against Jurkat and HL-60 cells, respectively (Table 2).

Table 2. Cytotoxicity Data for Selected Compounds^a

cell type	15	26	apicidin	SAHA
HL-60	8.2	2.8	0.28	0.93
Jurkat	12	8.1	0.83	1.1
HeLa	81	62	1.4	2.5
MCF-7	40	47	6.8	8.6
HEK293	>100 ^b	100	11	13

^aData represent growth inhibition (GI_{50}) values in units of μM . HL-60 (acute promyelocytic leukemia), Jurkat (T-cell leukemia), HeLa (cervical cancer), MCF-7 (breast cancer), and HEK293 (human embryonic kidney). Values are an average of two independent experiments performed in duplicate. ^b33% growth inhibition at 100 μM .

We further carried out Western blots for acetylated and total histone H3 to confirm that **15** and **26** act as HDAC inhibitors in cells. Indeed, we observed a dose-dependent increase in acetylated H3 in the presence of **15** and **26** (Figure S1 in the Supporting Information).

Enzyme kinetic analyses can provide valuable insights into the likely mechanism by which an inhibitor deactivates the enzyme. Accordingly, we measured progression curves for HDACs 1, 2, and 3 in the presence of varying concentrations of **26** to determine if it operated via a fast-on/fast-off or a slow-binding mechanism.^{23,24} The optimized procedure for the real-time enzyme-coupled assay used in these studies can be found in the Supporting Information.

Briefly, if the HDAC displays a constant rate of substrate turnover, there will be a constant rate of fluorescence increase (substrate deacetylation) over time, and the graph (over that time period) will be linear indicating a fast-on/fast-off mechanism of inhibition.^{23,24} An example of this type of inhibition is seen with **1** (SAHA)^{11,23} and, presumably, other hydroxamic acid-bearing HDAC inhibitors. However, if the rate of substrate conversion decreases over time (before the substrate has been significantly depleted), then the inhibitor can be described as having a slow-binding mechanism of action.^{23,24} From the data plotted in Figure 3a–c, it appears that **26** binds HDACs 1–3 with a fast-on/fast-off mechanism, thus rendering less likely the involvement of protein conformational changes or an allosteric mode of inhibition. Moreover, to establish whether substrate and inhibitor **26** binding to the enzyme were mutually exclusive events, we measured product formation under conditions of varying substrate and inhibitor concentration for HDACs 1 and 3, followed by a Lineweaver–Burk analysis (Figure S2 in the Supporting Information). We found that **26** fits the model for a competitive inhibitor for both HDACs. Thus, **26** appears to bind to the enzyme active site pocket in a similar manner as its progenitor, despite lacking the Zn^{2+} -binding side chain. Lastly, we determined the inhibition constant (K_i) for **26** with HDACs 1, 2, and 3. This was accomplished by using eq 1 for a fast-on/fast-off inhibitor, where v_p is the velocity in the presence of the inhibitor, v_a is the velocity in the absence of the inhibitor, $[I]$ is the concentration of the inhibitor, K_m is the Michaelis–Menten constant for the substrate against each HDAC (10 μM), and $[S]$ is the concentration of the substrate.²³

$$(v_p/v_a) = 1/\{[I]/(K_i(1+[S]/K_m)) + 1\} \quad (1)$$

The measured v_p/v_a values (Figure 3a–c and Table S2 in the Supporting Information) were fit to eq 1 using GraphPad Prism

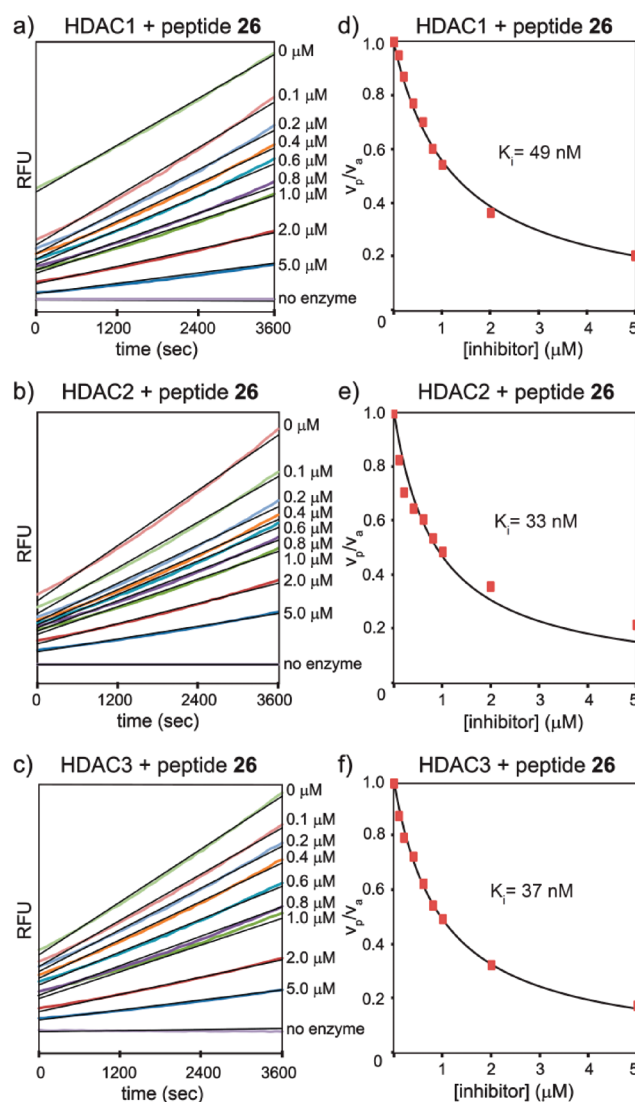


Figure 3. Progress curves for **26** shown as an average of two separate experiments performed each in duplicate. Panels a–c depict substrate turnover by increasing RFU (relative fluorescent units) over time with different inhibitor concentrations, $[I]$ (labeled next to each curve), for HDACs 1, 2, and 3, respectively. Panels d–f are plots of reaction velocity in the presence of inhibitor (v_p) over the absence of inhibitor (v_a) vs $[I]$ used to determine the K_i for **26** against HDACs 1–3.

to give the calculated K_i values for **26** against HDACs 1–3 of 49, 33, and 37 nM, respectively.

Although HDAC inhibitors are promising therapeutic agents for many diseases, acute dose-limiting toxicity and a narrow therapeutic index limit their widespread clinical utility for treating chronic diseases. We have developed inhibitors that lack the commonly used Zn^{2+} -binding group as a step toward mitigating these problems. We reported a macrocyclic peptide scaffold that does not require a Zn^{2+} -binding group for activity against HDACs 1, 2, and 3. Lead compounds **15** and **26** have translatable potency from in vitro experiments with recombinant enzymes to efficacy in cell-based assays. Our results provide the impetus for further research in advancing metal ion-binding independent classes of HDAC inhibitors and assessing their potential in drug design.

■ ASSOCIATED CONTENT

■ Supporting Information

Experimental procedures for compound synthesis, HDAC inhibition assays, cell growth assays, and kinetic assays as well as characterization data for new compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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