A Novel Series of Potent and Selective Ketone Histone Deacetylase Inhibitors with Antitumor Activity in Vivo

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Abstract: Histone deacetylase (HDAC) inhibitors offer a promising strategy for cancer therapy, and the first generation HDAC inhibitors are currently in the clinic. Entirely novel ketone HDAC inhibitors have been developed from the cyclic tetrapeptide apicidin. These compounds show class I subtype selectivity and levels of cellular activity comparable to clinical candidates. A representative example has demonstrated tumor growth inhibition in a human colon HCT-116 carcinoma xenograft model comparable to known inhibitors.

Aberrant regulation of gene expression is at the basis of many human diseases and notably many forms of cancer.[1] Yet attempts to pharmacologically target transcription complexes have been to date limited. In chromatin, DNA is wrapped around a core of eight histones to form nucleosomes, and these core histones have long N-terminal extensions that undergo extensive post-translational modifications including not only acetylation, methylation, and phosphorylation but also ubiquitination, sumoylation, and ADP-ribosylation.[2,3] The discovery that inhibition of chromatin modifying enzymes modulates transcription has allowed the development of novel pharmacologic agents.[4]

The acetylation status of lysine residues in these nucleosomal histone proteins is tightly controlled by two counteracting enzyme families, the histone acetyl transferases (HATs) and the histone deacetylases (HDACs).[a] The latter family is divided into four groups: classes I, II, and IV are closely related Zn-dependent enzymes, while the sirtuins, or class III, are structurally unrelated NAD-dependent deacetylases.[5] The presence of acetyl groups on these lysine residues neutralizes the positive charges of the histone tails, thereby decreasing interactions with DNA, relaxing the chromatin, and allowing access to transcription factors.[6] In contrast, removal of the acetyl groups from these Ac-lysines represses transcription by facilitating the interactions of ε-amino group with the DNA thereby condensing the chromatin. In addition to histones, a number of other proteins also undergo acetylation. Generally, acetylation has profound influences on their metabolic stability or biological function and thus can be regarded as a post-translational control mechanism of function.[7]

Histone deacetylase inhibitors (HDACi's) are emerging as a new class of anticancer agents and have been shown to alter gene transcription and exert antitumor effects such as growth arrest, differentiation, apoptosis, and inhibition of tumor angiogenesis.[7–9] However, the precise mechanism of action remains unclear.[7] Recently, vorinostat (**1**) (Zolinza, formerly known as SAHA) was approved for the treatment of the cutaneous manifestations of advanced CTCL that has relapsed or progressed on or following two or more systemic therapies.[10] In addition, there are several HDACi's in clinical trials, showing efficacy in hematological and solid malignancies.[11]

To date these HDACi's belong to several distinct structural classes (Figure 1).[8] Hydroxamic acids like **1**,[12] **2**,[13] and **3**[14] have been reported, although these are typically unselective HDACi's. The aminobenzamides **4**[15] and **6**[16] have advanced into studies in man, and more recently, bis-aryl derivatives like **5** have been disclosed as selective HDAC 1 and 2 inhibitors.[17] Other classes

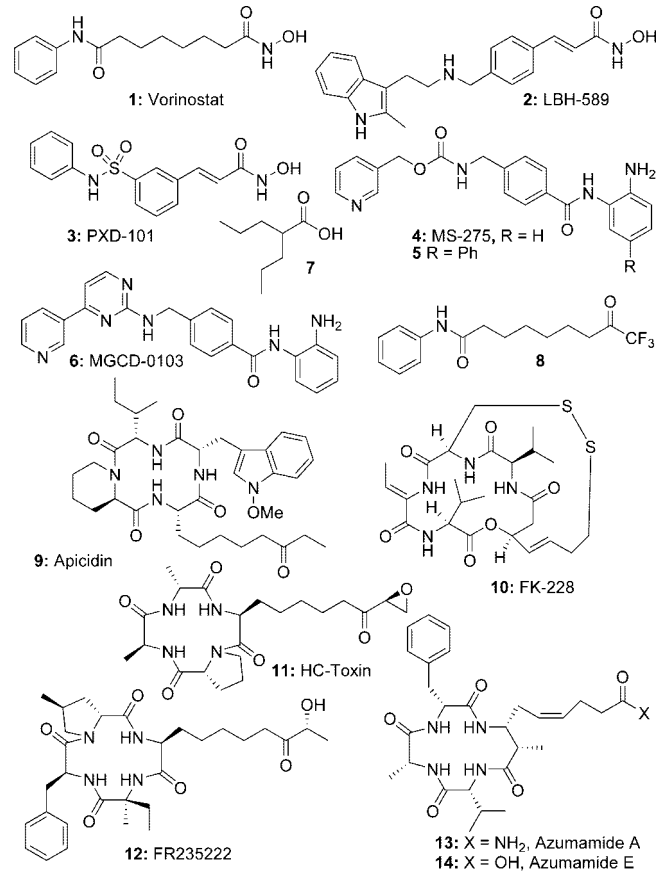
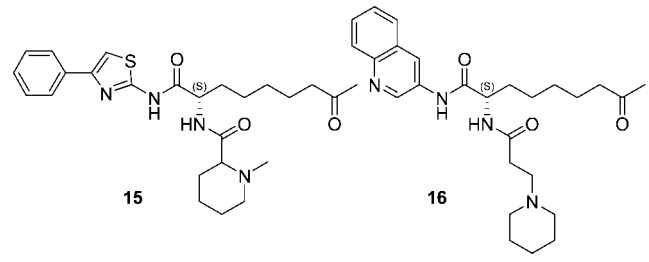


Figure 1. Known HDAC inhibitors.



	HDAC 1 IC_{50} (nM)	PRO(HeLa) IC_{50} (nM)
9	44	290
15	55	430
16	140	2,000

Figure 2. Activity of bis-amide HDAC inhibitors.[26]

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[a] Abbreviations: HAT, histone acetyl transferases; HDAC, histone deacetylases; HDACi, histone deacetylase inhibitor; ZBG, zinc binding group; HRE, human renal epithelial; MTD, maximum tolerated dose; TGI, tumor growth inhibition.

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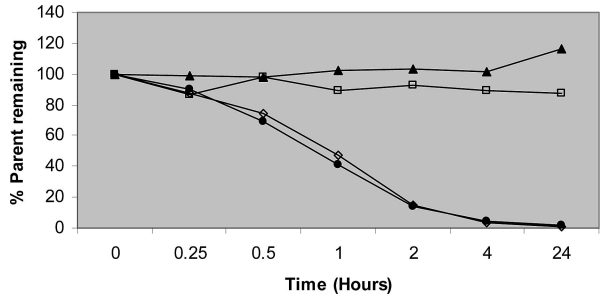


Figure 3. Plasma stability of **16** following incubations in mouse (◇), rat (●), dog (▲), and human (□) plasma for 24 h at 37 °C.

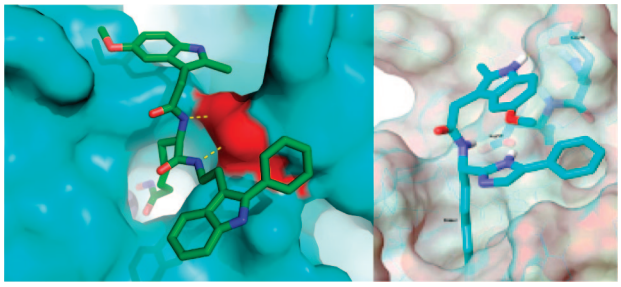


Figure 4. (A, left) X-ray crystal structure of HDAC 8 and bound inhibitor showing H-bonding to asp-101.[27] (B, right) Docking studies of HDAC 1 and **17** showing potential for H-bonding to asp-99 from the bioisoteric imidazole.

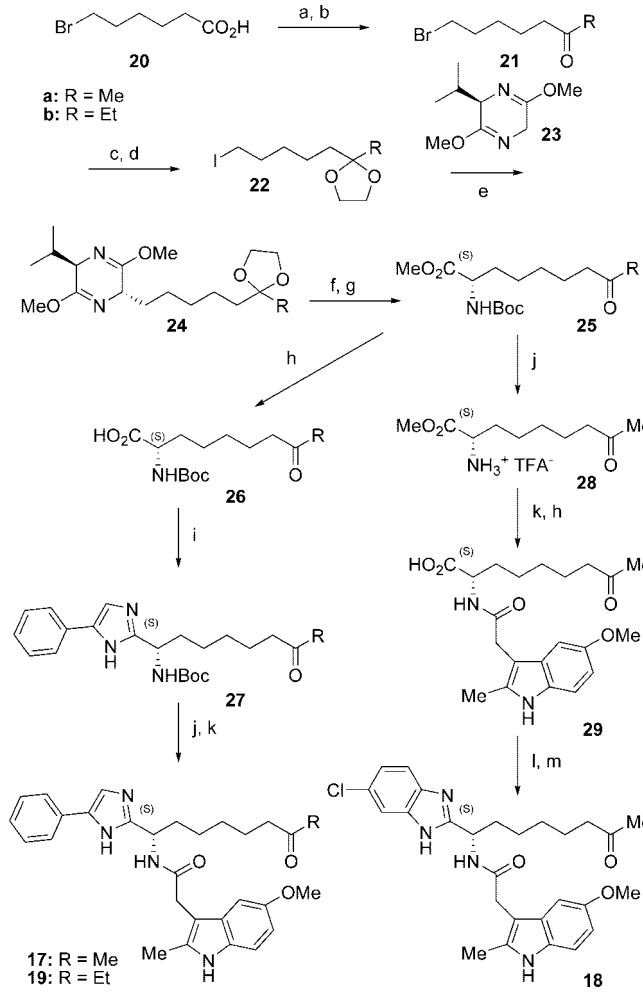
include short chain fatty acids, like valproic acid (**7**),[18] and electrophilic ketones such as **8**.[19] Furthermore, natural products are known to inhibit deacetylase activity, including among others apicidin (**9**),[20] FK-228 (**10**),[21] HC-toxin (**11**),[22] FR235222 (**12**),[23] and azumamide A and E (**13** and **14**),[24] and synthetic analogues have been described.[25]

Herein efforts to develop a novel class of HDACi's are described, targeting a second generation inhibitor with better potency and selectivity between HDAC isoforms.

Recently, initial efforts to that goal have been described whereby the natural product apicidin was selected as a suitable starting point.[26] This compound contains an ethyl ketone as potential zinc binding group (ZBG), a long alkyl chain, and the cyclic tetrapeptide that interacts with the surface of the HDAC. Despite the unusual ZBG, it is a relatively potent HDAC 1 inhibitor, $IC_{50} = 44$ nM, and displays good antiproliferation activity against cervical cancer HeLa cells, $IC_{50} = 290$ nM. Directed screening of the sample collection looking for compounds containing the unusual L-Aoda amino acid identified a more tractable lead which was optimized into potent, low molecular weight, selective, non-hydroxamic acid HDACi's exemplified by **15** (Figure 2). This compound inhibits HDACs 1, 2, 3, and 6 and shows good antiproliferative activity, for instance, HeLa cells $IC_{50} = 430$ nM.

In an effort to demonstrate in vivo antitumor activity with an HDAC inhibitor from this novel structural class, the microsomal stability of these compounds was determined. Unfortunately, **15** proved to be rapidly turned over in rat liver microsomes, and high intrinsic clearance in microsomes proved to be an issue with this class of compounds. The quinoline **16**, while maintaining enzyme activity, HDAC 1 $IC_{50} = 140$ nM, and low micromolar cellular activity PRO(HeLa) $= 2\ \mu$M, showed improved microsomal stability and was tested in vivo. Unfortunately, following iv administration in rats at 3 mg/kg **16** proved to have low exposure (AUC $= 0.05\ \mu$M·h) and to be cleared rapidly, Cl > 150 (mL/min)/kg. Further investigation

Scheme 1[a]



[a] Reagents and conditions: (a) (i) ClCOCOCl, DCM; (ii) MeOMe-$NH_2^+Cl^-$, Et_3N, DCM; (b) RMgBr, THF, 0 °C; (c) NaI, acetone, Δ; (d) $HO(CH_2)_2OH$, cat. PTSA, toluene, Δ; (e) **23**, BuLi, THF, −78 °C; then **22**, −78 °C to room temp; (f) H_3O^+/THF; (g) Boc_2O, $NaHCO_3$, dioxane/H_2O; (h) LiOH, THF/H_2O; (i) (i) Cs_2CO_3, EtOH; (ii) $PhCOCH_2Br$, DMF; (iii) NH_4OAc, xylene, Δ; (j) TFA, DCM; (k) (5-methoxy-2-methyl-1*H*-indol-3-yl)acetic acid, EDC, HOBT, Et_3N; (l) (i) ^{t}BuOCOCl, THF; (ii) $ClC_6H_3(NH_2)_2$, THF; (m) AcOH, 65 °C.

revealed that the compound was unstable in rat plasma, with the anilide bond being readily hydrolyzed (Figure 3). Similar observations were seen in mouse plasma, although the compound was stable in dog and human plasma. Further analogues from this bis-amide series of HDAC inhibitors were screened, and the vast majority was susceptible to hydrolysis. Given that good exposure in rodents would be a necessity for the development of these HDAC inhibitors, in order to perform efficacy and safety studies, attention therefore turned to replacement of the labile bond with a suitable bioisostere.

X-ray crystal structures of a related hydroxamic acid bound to HDAC 8 revealed that the two amide bonds of these inhibitors are involved in forming two hydrogen bonds to aspartic acid-101 on the rim of the binding pocket (Figure 4A).[27] This residue is conserved in all class I and II HDACs, with the exception of HDAC 11, and is believed to be crucial for substrate recognition. Replacement bioisosteres were designed to maintain this interaction. Accordingly 4-phenylimidazole **17** and the corresponding benzimidazole **18** were targeted. Both compounds should be capable of forming H-bonds to this aspartic acid present at the mouth of the binding cavity as shown by docking studies (Figure 4B).

Table 1. Activity on the HDAC Isoforms, IC_{50} (nM)[a]

compd	HDAC 1	HDAC 2	HDAC 3	HDAC 4GOF[28]	HDAC 4WT[28]	HDAC 5	HDAC 6	HDAC 7	HDAC 8
1	30	82	57	540	>10 μM	40% inh at 10 μM	43	>10 μM	1700
4	120	250	400	>10 μM	>10 μM	>10 μM	>10 μM	>10 μM	>10 μM
17	59	110	120	6000	>10 μM	>10 μM	340	>10 μM	>5 μM
18	670	880	1000	4200	>10 μM	>10 μM	970	>10 μM	>10 μM
19	26	59	48	>10 μM	>10 μM	>10 μM	40% inh at 10 μM	>10 μM	40% inh at 10 μM

[a] IC_{50} values are averaged from multiple determinations ($n \geq 2$), and the standard derivations are <30% of the mean.

Table 2. Antiproliferation Activity against Various Cell Lines, IC_{50} (nM)[a]

compd	cervical HeLa	colon HCT116	lung A549	kidney G401	ovarian A2780	human renal epithelial cells
1	460	1000	1800	1000	2600	14000
4	1800	700	1200	1300	3000	>20000
17	880	500	6300	960	2200	16000
18	3100	4200	8200	7500	11000	41000
19	340	330	5200	250	420	5300

[a] IC_{50} values are averaged from multiple determinations ($n \geq 2$), and the standard derivations are <30% of the mean.

The desired compounds were readily prepared as shown in Scheme 1 from the key *N*-Boc amino acid ester **25**. This was prepared using Schöllkopf chemistry, whereby alkylation of the protected alkyl iodide **22**, readily synthesized without chromatography, gave protected amino acids **25** in 58% yield. Hydrolysis of the methyl ester and alkylation with the bromoacetophenone gave the key cyclization precursor. Refluxing this keto-ester in xylene in the presence of a large excess of ammonium acetate gave rise to the desired 4-phenylimidazole **27**. Finally, deprotection and coupling yielded **17**. In a similar manner, the benzimidazole **18** was prepared from **25** by Boc-deprotection and coupling to the substituted indol-3-ylacetic acid. Subsequent hydrolysis and coupling of the resulting acid to 4-chloro-1,2-benzenediamine followed by cyclization with AcOH at 65 °C gave **18**.

Both compounds maintained good HDAC 1 inhibition (Table 1), with the 4-phenylimidazole **17** showing approximately 10-fold better activity than benzimidazole **18**, with $IC_{50} = 59$ and 670 nM, respectively. The improved activity of **17** was recapitulated on the other class I subtypes where **17** displayed 8-fold higher potency than **18** on HDAC 2 ($IC_{50} = 110$ vs 880 nM) and HDAC 3 ($IC_{50} = 120$ vs 1000 nM). As observed previously in the bis-amide class of HDACi's,[26] **17** proved to be a selective HDACs 1, 2, and 3 inhibitor, with weaker HDAC 6 activity and no inhibition on HDACs 4, 5, and 7 at 10 μM. Furthermore, **17** gave no inhibition of HDAC 8 at 5 μM, in contrast to **1** with $IC_{50} = 1.7$ μM.

With these encouraging enzymatic inhibitory properties, their cellular activity against a broad panel of human cancer cell lines was evaluated, including cervical, colon, kidney, ovarian, and lung cancer cell lines (Table 2). While **17** displayed submicromolar antiproliferation activity against HeLa, HCT116, and G401 cancer cell lines, $IC_{50} = 880$, 500, and 960 nM, the ovarian A2780 and lung A549 cell lines proved to be more resistant, and micromolar activity was observed. These results mirror the data seen with the prototypical HDAC inhibitors, **1** and **4**, where the A2780 and A549 cell lines are more resistant. In contrast, the benzimidazole **18**, in line with the reduced enzyme activity, displayed weaker antiproliferative effects with IC_{50} values in the low micromolar range on the panel of cell lines. All the HDACi's showed good selectivity for tumor over normal cells, as demonstrated by the weaker growth inhibition seen against human renal epithelial (HRE) cells. For instance, **1** and **17** have average IC_{50} values of 1.4 and 1.9 μM against the five tumor cell lines, while they display IC_{50} values of 14 and 16 μM on HRE cells.

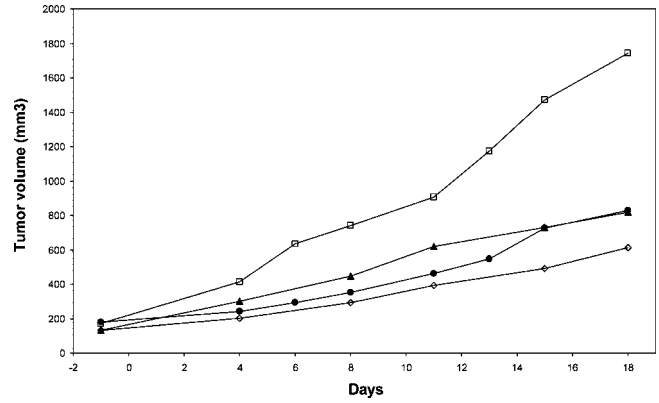


Figure 5. Human HCT116 colon cancer xenograft model in nude mice (15 per group): **1** (▲), **4** (◇), **17** (●), and vehicle (□) administered ip once a day, 5 day/week.

Given the instability seen with **16** and related compounds in rodent plasma, **17** and **18** were subjected to plasma stability studies. In contrast to the bis-amides, both displayed excellent stabilities and no degradation was seen at 37 °C in mouse, rat, dog, or human plasma for 4 h. Despite the finding that both compounds were turned over rapidly in rat and human liver microsomes, $Cl_{int} > 400$ (μL/min)/mg P, given the interest in this new class of inhibitors **17** was subjected to a rat PK study. Unsurprisingly, given the high microsomal turnover, **17** showed high clearance in vivo Cl = 80 (mL/min)/kg, short terminal half-life $t_{1/2} = 1.3$ h, and modest oral bioavailability $F = 15\%$.

With the encouraging cellular activity and acceptable rat pharmacokinetic profile, it was decided to evaluate **17** in an in vivo efficacy study, a human colon HCT-116 carcinoma xenograft model. This novel class of HDACi was profiled alongside the benchmark compounds **1** and **4**. In preliminary studies, the maximum tolerated dose (MTD) of all three compounds was determined following intraperitoneal once daily dosing for 1 week, as defined by no mortality and body weight loss less than 10%. MTD was established to be 100 mg/kg for **1**, 25 mg/kg for **4**, and 60 mg/kg for **17**. A quantity of 4×10^6 cells were inoculated subcutaneously in the flank of female mice, and tumors were allowed to grow for 14 days to an average size of 150 mm^3 prior to the initiation of treatment. Animals were then treated daily intraperitoneally at the MTD for 5 days/week for $2^1/_2$ weeks. Dose dependent tumor growth inhibition (TGI) was seen with **17** (Figure 5). In fact, at 60 mg/kg, **17** showed similar levels of tumor growth inhibition (TGI = 59%) compared to **1** and **4** dosed at their MTDs (TGI = 56% and 69%, respectively). These doses were well tolerated, and less than 10% body weight loss was observed with all compounds. To our knowledge this is the first example of a small molecule alkyl ketone HDACi demonstrating efficacy in xenograft studies.[29]

Encouraged by this result and the observation that alkyl ketones are indeed able to cause tumor growth inhibition in vivo,

further investigation into the zinc binding group was conducted, prompted by the observation that apicidin contains an ethyl rather than methyl ketone. The homologue of **17**, the ethyl ketone **19**, was prepared as previously and profiled. Interestingly, **19** was shown to be 2- to 3-fold more potent on HDACs 1, 2, and 3 and also in antiproliferation assays, compared to **17**. Noticeable was the finding that while **19** showed more activity on HDACs 1, 2, and 3, a loss of activity was seen on HDACs 4 and 6. Indeed, **19** showed only modest inhibition of HDAC 6 at 10 μM, whereas the corresponding methyl ketone **17** displays $IC_{50} = 340$ nM. It appears that this homologation to the ethyl ketone improves potency on HDACs 1−3 but also confers class I selectivity to this series of inhibitors. Further chain extensions result in significant erosion in enzyme activity (data not shown). Given the improved inhibition, it is not unsurprising that **19** demonstrates submicromolar antiproliferation activity on the panel of cell lines and indeed shows superior activity compared to **1** and **4**, notably the kidney G401 and ovarian A2780 cells.

In conclusion, an entirely novel class of ketone small molecule selective HDAC inhibitors have been developed. These compounds show levels of cellular activity comparable to existing clinical candidates. Furthermore, variations in the ketone zinc binding group have been demonstrated to fine-tune the isoform profile, thereby abolishing activity on HDAC 6, thus yielding selective HDAC 1, 2, and 3 inhibitors. A representative example **17** has been demonstrated to cause tumor growth inhibition in a xenograft model.

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Supporting Information Available: Complete experimental procedures and characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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