Novel Aminophenyl Benzamide-Type Histone Deacetylase Inhibitors with Enhanced Potency and Selectivity

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Abstract: Significant effort is being made to understand the role of HDAC isotypes in human cancer and to develop antitumor agents with better therapeutic windows. A part of this endeavor was the exploration of the 14 Å internal cavity adjacent to the enzyme catalytic site, which led to the design and synthesis of compound **4** with the unusual bis-(aryl)-type pharmacophore. SAR studies around this lead resulted in optimization to potent, selective, nonhydroxamic acid HDAC inhibitors.

Reversible histone acetylation is regulated by the opposing activities of histone acetyltransferases (HATs[a]) and histone deacetylases (HDACs). It plays an important role in the dynamic regulation of the chromatin structure and is essential for biological processes. The acetylation status of histones is crucial in modulating gene expression and cell fate,[1] and its misregulation is involved in the development of several cancers.[2] In general, histone deacetylation is correlated with transcriptional repression, whereas histone hyperacetylation facilitates gene expression.[3] Thus, inhibitors of HDAC activity induce histone hyperacetylation, leading to the transcriptional activation of suppressed genes, which are associated with cell cycle arrest, differentiation, or apoptosis in tumor cells. In recent years, inhibition of HDACs has emerged as a valid strategy to reverse aberrant epigenetic changes associated with cancer, and several classes of HDAC inhibitors have been found to have potent and specific anticancer activities in preclinical and clinical studies.[4] This is exemplified by compound **1** (Figure 1), which was launched in the U.S. market in October 2006 for the treatment of cutaneous T-cell lymphoma.

Histone deacetylases constitute an ancient family of enzymes that can be divided into four groups denoted as classes I−IV.[5] Class I enzymes (HDACs 1, 2, 3, and 8) are ubiquitously expressed, predominantly nuclear, and mainly function as transcriptional co-repressors. Class II HDACs (4, 5, 6, 7, 9, and 10) are selectively distributed among tissues, suggesting distinct functions in cellular differentiation and developmental processes.[6] Sirtuins, or class III, are structurally unrelated NAD-dependent deacetylase enzymes.[7] Finally, HDAC-11, another recently identified member of the HDAC family, bears low similarities with HDAC class I and class II and therefore is classified as a class IV. Classes I, II, and IV are closely related zinc-dependent enzymes bearing a highly conserved catalytic

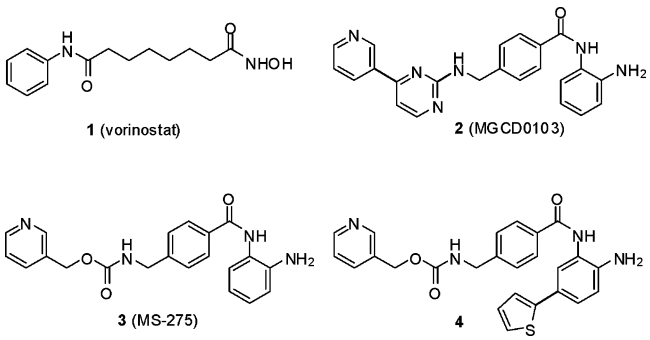

Figure 1.

domain. X-ray structural information on the zinc-dependent HDACs is available for two bacterial deacetylases, histone deacetylase-like protein (HDLP)[8] and histone deacetylase-like aminohydrolase (FB188 HDAH),[9] as well as human HDAC-7 and **8**.[10] In both bacteria and human HDACs, X-ray crystallography revealed a tube-like channel which leads to the active site in which the zinc ion is coordinated to side chains of two aspartate and one histidine residues. The second coordination sphere includes tyrosine and two histidine−aspartate dyads, which coordinate the hydrolytic water molecule (in some class II enzymes, that tyrosine is replaced by a histidine, and in one dyad, asparagine replaces aspartate).

Immediately below the active site, there is an internal cavity (14 Å cavity, Figure 2), originally described as being flexible and lined primarily with hydrophobic residues. The function of this cavity is not clear. This internal cavity was described for HDLP, FB188 HDAH, and hHDAC-8. Obviously, it is of considerable interest for inhibitor design as this space can potentially be used for additional binding interactions.[11]

As typical zinc hydrolases, most of the previously reported HDAC inhibitors are hydroxamic acids,[12] exemplified by **1**.[13] However, hydroxamate HDAC inhibitors, with the exception of a few compounds such as tubacin,[14] are nonselective inhibitors targeting both HDAC classes I and II. We identified nonhydroxamate HDAC inhibitors featuring the 2-aminobenzanilide functionality.[15] Unlike the hydroxamates, this class of inhibitors, typified by our clinical compound **2**, demonstrated very interesting isoform selectivity targeting primarily HDAC-1 and 2, with weaker activity against 3 and 11. Compound **2** is being tested in multiple phase II single-agent clinical trials, and objective clinical activity has been documented in acute myelogenous leukemia (AML), myelodysplastic syndrome (MDS), diffuse large B-cell lymphoma (DLBCL), follicular lymphoma, and Hodgkin's Lymphoma (HL). Other compounds have been identified which belong to the same class, such as **3**, currently in phase II clinical studies.

We built a homology model of HDAC-1 around the published coordinates of HDLP.[8] Compound **3** was docked into the active site, with the presumption that the carbonyl−oxygen and the *ortho*-NH_2 group coordinate to the active site zinc in a seven-membered ring. Examination of the docked structure revealed the presence of the cavity, mentioned above in the HDLP crystal structure, in fortuitous alignment with the para position of the pharmacophoric NH_2 (Figure 2). The structure suggested that substituents at the para position would fit snugly into this hydrophobic 14 Å internal cavity.

Therefore, the strategy for the further development of isoform-selective HDAC inhibitors was substitution of the aforemen-

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[a] Abbreviations: HAT, histone acetyltransferase; HDAC, histone deacetylase; HDLP, histone deacetylase-like protein; HDAH, histone deacetylase-like aminohydrolase; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)-phosphoniumhexafluorophosphate; AMC, aminomethylcoumarin.

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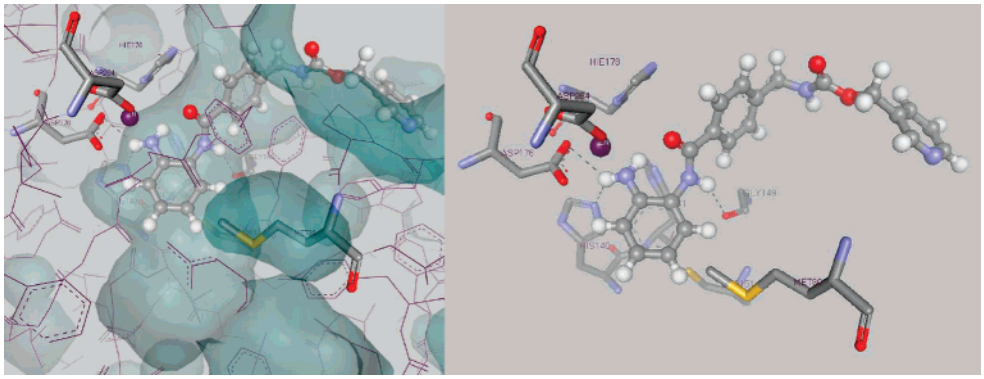
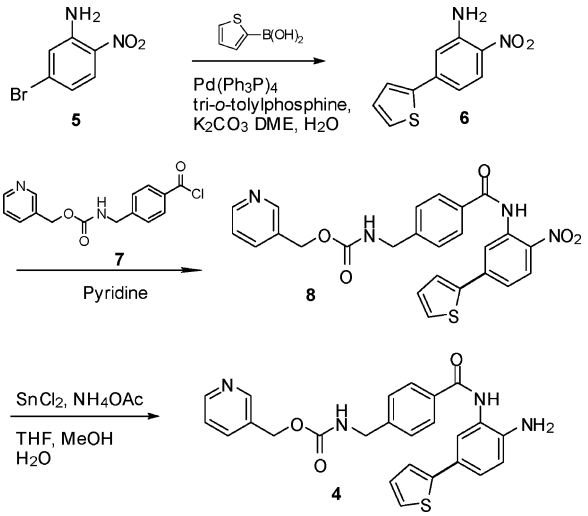


Figure 2. Human HDAC-1 homology model with the inhibitor **3** docked in the active site pocket, with its NH_2 group placed near the Zn atom (purple sphere) and bound to His 140 and 141 through hydrogen bonds. The empty cavity below the anilide ring is part of the 14 Å pocket. Key amino acid residues are depicted in bold style.

Scheme 1

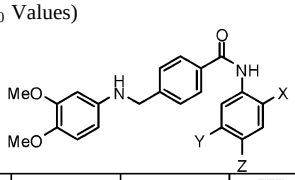


tioned para position with appropriate moieties. The presence of Met30 and Cys151 residues lining the walls of the internal cavity suggested placing of a thienyl substituent in order to benefit from potential sulfur interactions.[16] Thus, lead compound **4** was first synthesized.[17] As expected, the *para*-thienyl substitution picked up additional beneficial interactions with the enzyme, which resulted in 27-fold and 5-fold improvement in potency against recombinant human HDAC-1 and HDAC-2, respectively, compared with its parent analogue **3**. In addition to the gain in potency, we discovered an interesting change in the specificity profile. This class of novel benzamide inhibitors lost its activity against HDAC-3 and bore exclusively HDAC-1 and -2 inhibition. To our knowledge, this is the first HDAC inhibitor exhibiting such high isoform selectivity among class I enzymes.

These encouraging results led to further attempts to characterize the mentioned cavity. Therefore, a series of compounds bearing this novel 2-amino-5-arylanilide-based benzamide pharmacophore was synthesized according to Scheme 1 (exemplified by compound **4**).

Bis(aryl) core **6** was prepared by Suzuki coupling of 2-thiophene boronic acid with bromide **5**.[18] Masked dianiline **6** was then reacted with acid chloride **7**[19] to furnish nitroamide **8**. Reduction of the nitro group rendered 2-amino-5-thienylanilide **4**. Compounds **10**−**17** (Table 1) were synthesized similarly; however, in some cases, nitroaniline **6** was reacted with the corresponding acid by BOP[20] ((benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate)-promoted condensation in the presence of sodium hydride; compounds **16** and **17** were prepared starting from the appropriate 2-aminophenol, while the syntheses of reference compounds **9** and **16** have been previously described.[15]

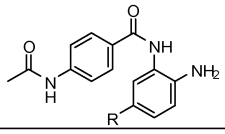
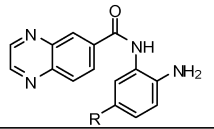
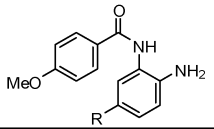
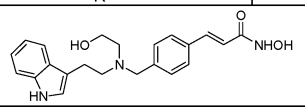
Table 1. Inhibition of Recombinant HDAC-1 and HDAC-2 (Representative IC_{50} Values)



Cpd.	X	Y	Z	HD-1 (μM)	HD-2 (μM)
3	-	-	-	0.54	0.5
4	-	-	-	0.02	0.1
9	NH_2	H	H	0.44	0.5
10	NH_2		H	0.05	0.08
11	NH_2		H	0.06	0.1
12	NH_2		H	0.05	0.06
13	NH_2		H	0.05	0.07
14	NH_2		H	>10	>10
15	NH_2	H		>10	>10
16	OH	H	H	0.3	0.2
17	OH		H	0.04	0.06

Consistent with the prediction above, adding an aryl group at the para position to the amine group significantly enhanced HDAC inhibitory activity against HDAC-1 and HDAC-2, up to 10-fold. Substituents like phenyl, 2- and 3-thienyl, and 2-furyl are well tolerated in this position, suggesting that hydrophobic rather than sulfur interactions in the 14 Å cavity are responsible for the increase in potency. Interestingly, replacing the NH_2 group in **9** and **10** by OH showed the same trend (compounds **16** and **17**, respectively). However, a bulkier benzo[*b*]thienyl (**14**) and substitution in the meta position to the NH_2 group (**15**) were not tolerated.

Table 2

Structure	R	Cpd	IC_{50} (μM)[a]				IC_{50} (μM)[b] in 293T cell	MTT IC_{50} (μM)[c]	
			HD1	HD2	HD3	HD8		HCT116	HMEC
	**H**	**18**	0.9	0.9	1.2	>20	6	4	>50
		**19**	0.04	0.1	>20	>20	0.3	0.5	>50
	**H**	**20**	2	2	>20	>20	3	11	>50
		**21**	0.06	0.2	>20	>20	0.9	0.9	26
	**H**	**22**	2	0.9	1.4	>20	3	10	>50
		**23**	0.05	0.2	>20	>20	0.15	0.4	>50
		**24** (LAQ -824)	0.008	0.007	0.009	0.4	0.05	<0.05	1

[a] Inhibition of recombinant class I HDACs. [b] Inhibition in whole-cell enzyme, in 293T cells transfected with vector alone. [c] MTT cytotoxicity/proliferation of human colon cancer HCT116 cells or human mammary epithelial cells HMEC (representative data).

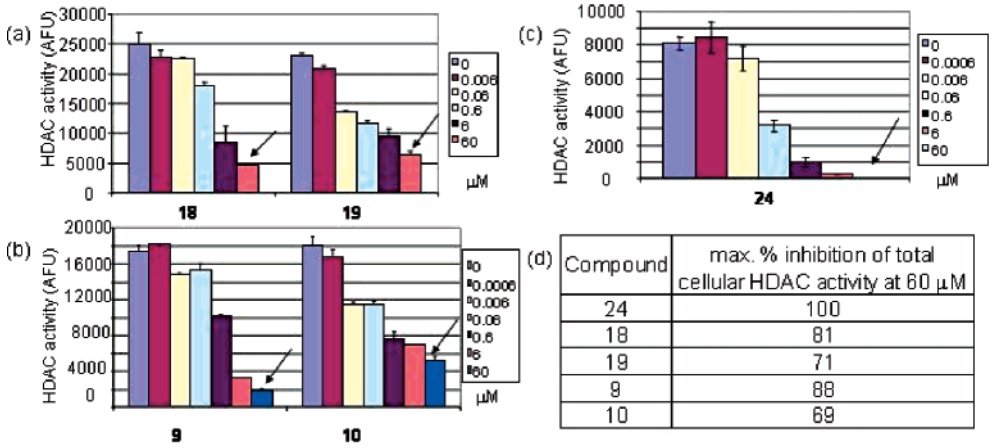


Figure 3. Dose-dependent inhibition of whole-cell HDAC inhibitory activity by selected thienyl benzamides, their parents (in a or b), and **24** (c) in human colon HCT116 cells; arrows indicate the maximum % of inhibition of total cellular HDAC activity at 60 μM, which is also shown in (d).

Importantly, the effect of the para substitution on the biological profile in the smaller HDAC inhibitors (Table 2) was even more dramatic. For example, 2-thienyl derivatives **19**, **21**, and **23** (Table 2) showed increased potency by 20- to 40-fold relative to the corresponding parent compounds **18** (Pfizer, CI-994), **20**, and **22**. Consistent with their increased potency against HDAC enzymes in vitro, thienyl compounds exhibited better antiproliferative activity than that of their parent benzamides against various human cancer cells. Moreover, there was no concomitant increase in cytotoxicity against normal human epithelial cells. Also, these compounds inhibited total HDAC activity in intact cells (293T), with demonstrated selectivity to class I HDACs. Furthermore, mechanistic studies are consistent with a competitive mode of inhibition (data not shown).

Compounds **18**−**23** were synthesized by BOP-promoted condensation between the corresponding commercial carboxylic acids and either 1,2-phenylenediamine or with compound **6**, following the same procedure described in Scheme 1. All benzamide-type compounds were screened in vitro against recombinant human HDAC isotypes 1, 2, 3, and 8 (Table 2), as well as 4, 5, 6, and 7, exhibiting inhibition lower than 50% when screened at a 20 μM concentration against these class II HDACs (data not shown). Whole-cell HDAC inhibitory activity was analyzed in 293T cells. The evaluation of in vitro antiproliferative activities was performed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay against a human cancer cell line (HCT116) and in human mammary epithelial cells (HMEC).

Isotype selectivity of HDAC inhibitors in intact cells can be estimated from the amount of inhibited HDAC activity relative to the total by using the pan-substrate Boc−Lys(acetyl)−AMC. For example, the hydroxamate pan-inhibitor Novartis' **24** was able to inhibit 100% of the total cellular HDAC activity (Figure 3c). On the other hand, benzamide inhibitors, which are known to be class I specific, inhibit about 90% of the total activity. In contrast, thienyl derivatives bring about only 75−80% inhibition of the total cellular HDAC activity (Figure 3a,b,d). This supports the results obtained from the recombinant enzymatic screening, which clearly indicate the selectivity of the para-substituted aminophenyl benzamides as highly specific inhibitors of HDAC-1 and HDAC-2.

In conclusion, the present study demonstrated the rational design of potent and selective HDAC inhibitors from available

structural information. The potential of this novel class of compounds for treating cell proliferation-related disorders, such as cancer, is currently being explored. Moreover, the novel isotype specificity, targeting only HDAC-1 and 2, should be instrumental in elucidating the mechanism(s) of HDAC involvement in cellular activities and in cancer.

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Supporting Information Available: Characterization of final analogues is available. This material is available free of charge via the Internet at http://pubs.acs.org.

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