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Toward isozyme-selective inhibitors of histone deacetylase as therapeutic agents for the treatment of cancer

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Abstract

Since post-translational modifications of proteins are key mechanisms for controlling cellular function, targeting the machinery involved in these modifications offers new opportunities for the development of therapeutic agents. The histone deacetylases (HDACs) represent an important family of enzymes that are involved in controlling the acetylation state of key lysine residues in histones and other proteins. The development of HDAC inhibitors for the treatment of several diseases, most notably cancer, has proceeded rapidly. Recent attention has turned towards the development of isozyme-specific inhibitors that will provide selective targeting. It is believed that the ability to target-specific HDACs rather than all family members will lead to superior therapeutics with better efficacy and lower toxicity. A review of recent patents shows that researchers are targeting a wide range of isozymes and that key advances in the structural biology of HDACs are providing important design information.

Keywords

Histone deacetylase: Also known as a lysine deacetylase, an enzyme that catalyzes the hydrolysis of an acetamide moiety derived from the ϵ -amino group of lysine; Histone: Highly basic proteins in eukaryotic chromatin involved in packaging of DNA into nucleosomes; Epigenetics: The study of phenotypic changes and alteration of gene expression induced by a modification of the genome (e.g., acetylation, methylation) rather than alteration of the genetic sequence; Isozymes: Highly related isoforms of an enzyme that have different sequences but catalyze the same biological reaction

Histone deacetylase (HDAC) enzymes affect many basic cellular processes related to differentiation and proliferation, thus, targeting these enzymes has recently been recognized as a new and successful strategy for the development of anticancer agents, which will be the focus of this review. HDAC inhibitors have shown a wide range of applications in several additional therapeutic areas, including inflammation [1], the treatment of epilepsy [2,3], and most recently, sickle cell anemia [4]. The effort to develop potent antineoplastic HDAC inhibitors culminated in the first-in-class approval of Zolinza® (generically known as vorinostat and suberoylanilide hydroxamic acid [SAHA]) by the US FDA in 2006 and Istodax® (generically known as romidepsin) in 2009, both for cutaneous T-cell lymphoma.

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HDAC inhibitors are believed to modulate gene expression by altering the acetylation status of lysine residues in the N-termini of **histones**, thus modifying the accessibility of the gene to cellular transcriptional machinery and producing **epigenetic** effects. The exact mechanism of HDAC inhibitors as anticancer agents is still an area of active investigation and may be related to apoptosis, cell cycle arrest or inhibition of DNA repair [5–7].

A total of 18 human HDAC **isozymes** are grouped into four classes. Class I enzymes include HDAC1, HDAC2, HDAC3 and HDAC8. With the exception of HDAC8, Class I HDACs are ubiquitously expressed in various human tissues, are located in the nucleus, and function as transcription corepressors. Class II enzymes, the expression of which is tissue-dependent, are further subdivided into Class IIa (HDAC4, HDAC5, HDAC7 and HDAC9) and Class IIb (HDAC6 and HDAC10). Class IIa HDACs have been shown to exhibit low enzymatic activity and have tissue-specific roles; Class IIb HDACs are uniquely characterized by the presence of two catalytic sites and are primarily involved in regulating protein folding and turnover. Class IV has a single member, HDAC11. Enzymes in Classes I, II and IV share a zinc-dependent catalytic mechanism; these classes will form the focus of this review. The remaining Class III HDAC enzymes, which are NAD⁺-dependent, are called the sirtuins.

The compounds currently in the clinic can be considered pan-HDAC inhibitors and exhibit some associated toxicity that may be related to the inhibition of the many functions of the different HDAC isoenzymes [8]. An attractive approach is the design of inhibitors for specific classes, or even isoforms of HDAC enzymes, in order to improve therapeutic effect and reduce toxicity [9–11]. Expression patterns of some of the individual HDAC isozymes in tumors may inform the selection of a particular isozyme target in a drug-discovery program [12,13]. For example, overexpression of HDAC2 has been linked to gastric cancer [14] and colon cancer [15], HDAC1–3 to ovarian cancer [16] and HDAC1 and HDAC3 with lung cancer [17]. Also, high levels of expression of HDAC1–3 in gastric cancer were associated with poor prognosis [18]. In contrast, experiments conducted with knockout mice and RNAi have revealed that deletion of HDAC3, HDAC9 and HDAC5 impairs cardiac function, implicating a possible role of these isozymes in cardiac health [19,20]. Additionally, expression of Class II HDAC4–7 and HDAC10 was associated with improved prognosis in non-small-cell lung cancer [21].

Four crystal structures of human HDAC isozymes can be used to elucidate amino acid differences that will inform selective design. In 2004, Vannini *et al.* reported the crystal structure of HDAC8 bound to a hydroxamic acid inhibitor [22]. Independently, also in 2004, Somoza *et al.* reported the structure of HDAC8 bound to four hydroxamic acid inhibitors [23]. In 2008, Schuetz *et al.* determined the structure of the catalytic domain of HDAC7, a Class IIa enzyme, bound to two different hydroxamic acid inhibitors [11]. Also in 2008, Bottomley *et al.* reported the structure of the catalytic domain of HDAC4, another Class IIa enzyme, bound to trifluoromethylketone or hydroxamic acid inhibitors [24]. Finally, in 2010, Bressi *et al.* determined the structure of HDAC2 bound to an aminobiphenyl benzamide [25] that explores the ‘foot pocket’, composed of residues Y29, M35, F114 and L144. Comparison of the structures of these four isozymes reveals conservation of the Asp-His-Asp catalytic triad that coordinates the zinc ion [26]. Although studies have shown that HDACs are capable of binding more than one metal ion, and HDAC8 in particular may use iron as a metal cofactor *in vivo* [12,27], the crystal structures show zinc bound in the active site. There are distinct differences in the active sites (Figure 1) that may lend insight into selective inhibitor design. For example, the active site of HDAC7 is more constricted as the helix containing residues T625 and D626 (Y100 and D101 in HDAC8) and the loop with L810 are much closer to the inhibitor binding site. Conversely, the active site of HDAC4 is much more open when compared with HDAC7 and 8, as it does not possess the loop-containing residues 100–101 (HDAC8 numbering). HDAC8 possesses three residues that

differ from other isozymes: Tyr-100 is located at the opening of the active site, Y100 is located close to the hydroxamic acid moiety and Y306 is a leucine in HDAC4 and HDAC7. In addition, the crystal structures can be used to elucidate differences between closely related isozymes, even when experimentally determined structures are not available. For example, the structure of HDAC2 superimposed with the predicted structure of HDAC1 shows that there are significant differences in the conformation of loop residues H28–K31 in HDAC1 and the foot pocket residues Y29–M35. These important structural differences are likely to influence the design of selective inhibitors.

HDAC inhibitors in clinical development

There are four major chemotypes of HDAC inhibitors currently in clinical development: hydroxamic acids, short-chain fatty acids, cyclic tetrapeptides and benzamides (see Figure 2 for all compound structures). All inhibitors share a common pharmacophore pattern consisting of a zinc-binding domain, a linker domain that mimics the substrate and occupies the active site channel, a connecting unit, and a capping unit that contacts the surface of the enzyme. Hydroxamates are the most extensively investigated and promising class of HDAC inhibitors [2,28,29]. With the exception of the HDAC8-selective PCI-34051 [30] and the HDAC6-selective Tubacin (tubulin acetylation inducer) [31–33], all hydroxamates are considered to be pan-HDAC inhibitors [29]. New hydroxamates in clinical trials [29] include belinostat for T-cell lymphoma and leukemia, and panobinostat [34] for Hodgkins' lymphoma, multiple myeloma and acute myeloid leukemia [35].

The cyclic peptides show preferential inhibition of Class I HDACs [1,12]. Romidepsin is a bicyclic depsipeptide antibiotic isolated from the bacterium *Chromobacterium violaceum* that shows anticancer activity [29,36]. Romidepsin was approved by the FDA in 2009 for the treatment of cutaneous T-cell lymphoma in patients who have received at least one prior systemic therapy; it is currently undergoing development in monotherapy and in combination therapy for the treatment of both solid and hematological cancers [36]. Apicidin is a cyclic peptide that shows selectivity towards Class I HDACs, but is yet to undergo any clinical development [37]. Entinostat (otherwise known as MS-275) and mocetinostat [38], both synthetic benzamide derivatives, have been shown to selectively inhibit HDAC1/HDAC3 and HDAC1/HDAC2, respectively. Both entinostat and mocetinostat are currently in clinical trials for monotherapy and combination therapy for the treatment of both solid and hematological cancers [2,29,39]. Short-chain fatty acids, most notably valproic acid (VPA), have been shown to weakly inhibit both Class I and II HDACs with IC₅₀ values in the millimolar range and is used in the treatment of seizure and bipolar disorders. VPA has recently shown promise in the treatment of both solid and hematological malignancies [12,40]. The mechanism of the anticancer effects of HDAC inhibitors is not well understood; however, it is postulated that HDAC inhibitors may activate altered gene expression through histone acetylation as well as altered function of proteins involved in the regulation of cell-cycle progression and cell death [2]. For example, VPA has been shown to specifically induce proteasomal degradation of HDAC2 via ubiquitinconjugating enzyme and the ubiquitin protein ligases RLIM [12].

Although there are a few clinical trials ongoing with isozyme-specific inhibitors, emerging data suggest that these inhibitors have reduced toxicity relative to the pan-HDAC inhibitors. Specifically, QT prolongation was reported as a dose-limiting toxicity in trials with pan-HDAC inhibitors [41]. Results from trials with mocetinostat show that grade 3 and 4 toxicities mainly include fatigue. Furthermore, hematological toxicity is rare and QT interval changes were not observed [39,41].

Considerable commercial interest has centered on the development of isozyme-specific HDAC inhibitors in recent years. As background, a patent review summarizing the general development of HDAC inhibitors from 2007 until mid-2009 was published in 2009 [42]. This review will focus specifically on patents that describe the development of compounds directed towards the isozyme-selective inhibition of HDACs. We will also focus on antineoplastic applications and will cover the period 2007–2011.

Class I-specific inhibitors

Shenzhen Chipscreen Biosciences, Ltd, claim a series of 2-indolinone derivatives as selective HDAC inhibitors for treatment of a wide range of diseases, including leukemias and other malignancies [101]. The compound class is based on an isatin-like framework with an arylalkylidene linked through a flexible, saturated linker to a 2-aminobenzamide head group. These compounds generally show preferential inhibition for Class I HDACs with differential preference over the Class IIa HDACs (HDAC4, 5 and 7). A representative compound **1** potently inhibits Class I enzymes (97%) and HDAC4/5 (85%) with significantly less inhibition of HDAC7 (35%) (Table 1). Evaluation of compound **1** against four cell lines shows activity, measured using the concentration of drug that is required to inhibit cell growth by 50% (GI₅₀), against lymphoma (Hut-78, GI₅₀ ~ 4 μ M) and leukemia (HL60, GI₅₀ ~ 3 μ M) with weaker activity against cervical (HeLa, GI₅₀ ~ 11 μ M) and lung (A549, GI₅₀ ~ 20 μ M) lines.

A series of novel 2-aminobenzamides linked to a key benzofuran unit through a flexible linker are disclosed by Gilead Sciences, Inc. as selective agents for the treatment of Class I-dependent diseases [102]. A preferred embodiment, compound **2**, shows IC₅₀ values for HDAC1 of 0.03 μ M (without pre-incubation) and 0.0033 μ M (with pre-incubation). Conversely, HDAC6 is inhibited at concentrations greater than 50 μ M, regardless of incubation protocol. Merck scientists disclose a series of novel hydroxamates containing a key sulfonamide linkage (**3**) for use against a variety of proliferative diseases [103]. Specific compounds in this series are shown to be selective inhibitors of Class I HDACs, with a focus on HDAC1. All compounds are shown to have IC₅₀ values less than 100 nM against HDAC1 with some compounds having IC₅₀ values lower than 10 nM. Compounds in this series are also shown to have activity against a leukemia line (SC9) with GI₅₀ values less than 1 μ M.

Class IIa-specific Inhibitors

Harvard College and Dana-Farber Cancer Institute filed a patent describing novel cinnamic hydroxamates [104]. The inventors used a combination of sequence alignment and examination of the structures of HDAC4, HDAC7 and HDAC8 to design and disclose Class IIa enzyme (HDAC4, 5, 7 and 9) inhibitors [43]. As such, while the inventors claim that the compounds may be effective in treating proliferative diseases such as cancer, it is also claimed that the compounds may be effective in treating diseases associated with aberrant Class IIa enzyme activity, such as transplant rejection as well as autoimmune and vascular diseases. Several cinnamic hydroxamates, out of a total of 320 that were synthesized and evaluated, are potent and selective for Class IIa enzymes over Class I enzymes; four are specifically depicted in the patent (**4–7**). In general, it appears that para-substituted cinnamic hydroxamic acids (e.g., **8**) show increased potency in assays with HDAC5 (IC₅₀ value range of 18 nM to 5 μ M depending on the capping feature), relative to meta-substituted compounds (e.g., **9**). The inventors also emphasize that a completely nonselective compound may be useful for research purposes. In their screens, they identified such a compound and named it Pandacostat® (**10**); it inhibits all profiled HDAC isoforms equally with inhibition constant (K_i) values of 0.1–1 μ M for HDAC1–9.

Harvard College and Dana-Farber Cancer Institute also claim a series of fluorinated cinnamic hydroxamates useful both as research tools and therapeutic agents [105]. The compounds of the invention, while not selective for Class IIa isozymes over Class I, are intended to display increased potency for Class IIa isozymes. Specifically, while panobinostat shows activity against Class IIa isozymes (0.5–5 μM), a fluorinated analog of the compounds (**11**) shows a five- to ten-fold increase in activity. Furthermore, the acidic fluorohydroxamic acid MAS1702 (**12**) inhibits Class IIa HDAC enzymes 3.5–5-fold better than the less acidic analog MAZ1704 (**13**).

HDAC1- & HDAC2-selective inhibitors

Georgetown University claims a series of hydroxamates and mercaptoacetamides containing various aromatic and heteroaromatic substructures that function as selective Class I inhibitors [106]. The compounds were evaluated as enzyme inhibitors and for antiproliferative effects against a variety of cell lines, including PC-3 (prostate cancer), MCF-7 (breast cancer), HeLa (cervical cancer) and SQ-20B (squamous carcinoma). Overall, the hydroxamate derivatives are significantly more active than the corresponding mercaptoacetamides with GI_{50} values ranging from 0.1 to $>60 \mu\text{M}$ against these cell lines. A derivative optionally substituted with a phenylalanine (**14**) is particularly active (GI_{50} values ranging from 3 to 8 μM) as compared with SAHA. However, initial evaluation against the enzymes shows little selectivity within the Class I isoforms. Further modification of the leads led to the discovery of inhibitors with a phenylthiazole moiety that display much higher levels of selectivity within Class I. Compounds **15**, **16** and **17** are notable in inhibiting HDAC1 and HDAC2 completely at a concentration of 0.5 μM . At this same concentration of inhibitor, 30–50% activity remains for both HDAC3 and HDAC8. Excitingly, compounds **15–17** also show antiproliferative effects for the previously described cell lines at concentrations less than 1 μM .

Georgia Tech Research Foundation reports a series of nonpeptide macrocyclic HDAC inhibitors containing a glycosylated macrolide ring linked through a flexible spacer to a zinc-binding group [107]. Although macrocyclic topologies have been well known for HDAC inhibitors, they have always been based on a peptidic core. These peptidic compounds raise obvious concerns for stability and oral bioavailability and sample a fairly limited range of chemical space. The inventors explored a nonpeptidic (polyketide) scaffold based on well-known antibiotics. After evaluation in enzyme-inhibition assays, it is apparent that activity is dependent on spacer length, with six methylene spacer units (**18**) most potent. Compound **18** inhibits HDAC1 and HDAC2 with an IC_{50} value of 1.03 nM, HDAC8 with an IC_{50} value of 544.6 nM (528.7-fold difference) and HDAC6 with an IC_{50} value of 728.7 nM (707.5-fold difference). Longer spacers show a reduction in overall HDAC inhibitory activity and selectivity. Further evaluation of compounds with varying spacer lengths against prostate (DU-145), lung (A549) and breast (MCF-7) cancers show that compound **18** is most potent with GI_{50} values of 0.66 μM (against A549), 0.75 μM (against MCF-7) and 0.82 μM against DU-145. There is no measurable toxicity against normal cells at concentrations of 10 μM .

HDAC1-selective inhibitors

Arqule, Inc. claim a series of aliphatic hydroxamates that were not designed to target a specific isoform [108]. However, several of these hydroxamates are comprehensively evaluated against HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, HDAC6, HDAC8 and HDAC9 to probe for the origins of isozyme selectivity. Some notable compounds, compounds **19** and **20**, include an interesting tricyclic system tethered to a hydroxamate through a flexible aliphatic linker. In general, these compounds show high potency towards

several of the isozymes in the panel (e.g., IC_{50} value of 22 nM against HDAC1) but with some selectivity over HDAC2 (IC_{50} 1.3 μ M) and HDAC8 (IC_{50} 0.5 μ M). Additionally, the compounds show very good growth inhibition of several transformed cell lines including HeLa, HCT-116 and Hut78 (GI_{50} value range = 0.02–0.5 μ M).

HDAC4-selective inhibitors

A heterocyclic series of trifluoroethanone derivatives are claimed by Merck as selective HDAC inhibitors [109]. The example trifluoromethylketone (**21a**) shows decent selectivity towards HDAC4 (IC_{50} = 95 nM) over HDAC1 (IC_{50} = 710 nM) and HDAC6 (IC_{50} = 350 nM). Given that many HDACs, such as SAHA and trichostatin A, show poor inhibitory activity towards HDAC4, these trifluoroethanone derivatives are promising novel HDAC inhibitors. Interestingly, substitution of the trifluoromethylketone with the hydroxamate (**21b**) dramatically changes the activity and is more reflective of SAHA with a loss in HDAC4 activity with concomitant increase in potency against HDAC1 and HDAC6, both in the low nanomolar region.

HDAC6-selective inhibitors

HDCA6 is a somewhat unique isozyme among the zinc-dependent members in that it has two functional catalytic domains and a carboxy-terminal zinc-finger that binds to misfolded, ubiquitinated protein. In addition to histones, some of its known substrates include - tubulin, -catenin, HSP90, cortactin and peroxiredoxins [44,45]. Interestingly, one of the key nonhistone substrates of HDAC6 is the heat-shock protein HSP90 that serves to properly fold a variety of proteins, including the oncogenic fusion protein BCR-ABL [46]. Inhibition of HDAC6 function leads to hyperacetylation of HSP90 and subsequent loss of chaperone activity. In the absence of HSP90-mediated folding, the client proteins are subsequently degraded by the proteasome [46]. In a related finding, Lin *et al.* studied the downregulation of a different oncogenic client protein, KIT, upon treatment with the pan-HDAC inhibitor AR-42 [47]. These authors found that decreased levels of KIT partly resulted from a reduction in c-KIT transcription. However, the studies also showed that KIT downregulation was attributed to suppressed HSP90 activity, inferred from upregulation of inducible HSP70. However, treatment with AR-42 did not produce hyperacetylated HSP90, HSP70 or important co-chaperones, although tubulin hyperacetylation was evident. Presently, it is unclear if this HSP90 loss of function without hyperacetylation is unique to the inhibitor AR-42 or the mast cell line studied. It should also be noted that knockdown of HDAC6 has been shown to prevent HSP90 from forming a stable complex with the glucocorticoid receptor, although HSP90 retained the ability to participate in dynamic association [48]. HDAC6 is most likely not the only HSP90 deacetylase as studies with the drug FK228 have shown hyperacetylation of HSP90 independent of inhibition by HDAC6 [49–51]. Related research by Scroggins *et al.* on the effects of acetylation of mammalian HSP90 on K294 is consistent with the model whereby acetylation of HSP90 decreases affinity for most client proteins, specifically co-chaperones; this affinity is increased upon deacetylation [52]. Selective inhibitors targeting HDAC6 have been the focus of significant development over the past several years.

Brown *et al.* claim a series of HDAC inhibitors with fluorescent properties that can be used to “enable diagnostic and/or therapeutic applications, or to enable guided treatments (e.g., radiotherapy or surgery)” by monitoring HDAC activity [110]. These theranostic agents can be used for diagnosing a tumor, diagnosis followed by treatment via HDAC inhibition, or alternatively, determining the location of a tumor in order to direct additional therapy such as surgery or radiation [53]. The inventors emphasize the need for isoform-selective compounds that will act as therapeutic agents as well as biological probes. The compounds

are characterized by an optionally substituted naphthyl group or fused bicyclic heteroaryl linked through a sulfonyl to an alkyl chain and hydroxamic acid. The most potent of these compounds (**22** & **23**) inhibit HDAC6 with IC₅₀ values of 128.9 and 76.2 nM, respectively. For comparison, the same compounds inhibit HDAC1, 2, 3 and 8 with IC₅₀ values greater than 950 nM. The inventors show that the compounds inhibit the growth of prostate (PC3) cells at 1.538 μ M, androgen-independent prostate (C42) cells at 1.913 μ M, androgen-dependent prostate (LNCap) cells at 1.224 μ M, squamous cell carcinoma (SQ20B) cells at 6.425 μ M and breast cancer (MCF-7) cells at 9.199 μ M. Additionally, imaging shows that the compounds are localized to the cytoplasm and not the nucleus, yet they exhibit inhibitory effects on the acetylation status of the nuclear histones, suggesting that they may inhibit HDACs that shuttle between the cytoplasm and nucleus. Using antibodies against HDAC4, the inventors show that HDAC4 is trapped in the cytoplasm of prostate cancer cells, similar to SAHA. Additional imaging experiments show that the compounds inhibit tubulin and histone deacetylation. Finally, molecular modeling using the structure of HDAC8 to predict the structure of HDAC6 reveals that the region near the surface of the protein, immediately adjacent to the catalytic site, is unique to each isoform and that important selectivity-conferring interactions may be garnered in this region.

A series of novel hydroxamic acids designed to encumber isoform selectivity, specifically against HDAC6, are disclosed by The Board of Trustees of the University of Illinois [111]. HDAC6 is specifically targeted owing to its unique substrates, its relevance to both cancer and neurodegenerative disorders, and the fact that inhibition of HDAC6 shows minimal toxicity [54]. In addition to a hydroxamic acid and linker, these compounds contain a heterocyclic moiety, such as indole or carbazole, which is designed to interact with the surface of the HDAC isoform. Seven compounds falling into this class are profiled against HDAC1 and HDAC6 enzymes. Compounds **24** (tubastatin A) and **25** show potent inhibition of HDAC6 with IC₅₀ values of 0.014 and 0.0014 μ M, respectively, and show less potent inhibition of HDAC1 (13.8 and 5.18 μ M, respectively). Compound **24** is further profiled against all HDAC isozymes, exhibiting strong selectivity for HDAC6 (14 nM), moderate potency against HDAC8 (814 nM) and low potency against all other isozymes (>30 μ M).

Harvard College and Dana-Farber Cancer Institute disclose a series of selective compounds targeting HDAC6 and designed to inhibit the deacetylation of tubulin [112]. Compound **26** is specifically claimed and exhibits IC₅₀ values of 45 nM for HDAC6, compared with 240 nM for HDAC1, 150 nM for HDAC2, 390 nM for HDAC3 and 5000 nM for HDAC8. In a cellular assay, **26** exhibits a tubulin deacetylase IC₅₀ value of 0.35 μ M, compared with HDAC IC₅₀ value of 4.3 μ M, yielding a 12.3-fold selectivity. In combination, **26** and bortezomib exhibit synergistic effects on the proliferation of multiple myeloma cells.

An interesting series of inhibitors based upon a central 1,3-dioxane core is claimed by Schreiber *et al.* for application in a range of diseases [113]. Tubacin (referenced as JCWH114 in patent), a hydroxamate derivative, is shown to be a selective tubulin deacetylase inhibitor that exhibits high selectivity for HDAC6 (IC₅₀ ~ 4 nM) over other HDACs (IC₅₀ values are approximately 1000-fold higher). These results suggest that HDAC6 maintains tubulin deacetylase activity and that the anti-proliferative properties of tubacin are mediated through this pathway. The related carboxylate analog, compound **27**, does not affect histone or tubulin acetylation states. Although tubacin is a deacetylase inhibitor, it does not seem to affect global histone acetylation, gene expression or cell cycle.

A novel series of synthetic macrocycles are claimed by Sigma Tau as selective inhibitors of HDAC6, focusing on a range of therapeutic applications including cancer, inflammatory diseases, neurological disorders and parasitic infections [114]. The invention describes structurally novel nonpeptidic macrocyclic lactams positioned with pendant hydroxamates

and other zinc-targeting functionality. Three example compounds **28–30** show excellent levels of HDAC6 selectivity with IC₅₀ values for HDAC6 between 4.4 and 75 nM, and IC₅₀ values for the remaining ten isozymes ranging from 245 to 5333 nM. Interestingly, the stereochemistry at position six of the hexanoic acid chain does not affect potency.

A series of substituted tetrahydroisoquinoline and isoindoline-derived hydroxamic acids are disclosed by 4SC AG as selective HDAC6 inhibitors for use in the treatment of cancer [115]. As it is known that HDAC6 deacetylates tubulin and is co-localized with microtubules, the inventors emphasize a strategy to synergistically combine selective HDAC6 inhibitors with compounds that affect the mitotic spindle (e.g., Taxol™). A total of 24 compounds were synthesized and evaluated against HDAC1, 3, 6 and 8. These compounds show pIC₅₀ (-log IC₅₀) ranges of 4.72 to 6.52 against HDAC1, pIC₅₀ of 4.07 to 5.29 against HDAC3, pIC₅₀ of 6.26 to 8.57 (**31**) against HDAC6 and pIC₅₀ of 5.03 to 7.26 against HDAC8. Overall, the compounds in the series show the greatest inhibitory activity against the HDAC6 isoform, as evidenced by the higher pIC₅₀ values when compared with the other isozymes. The compounds were further evaluated against both solid tumor and hematological cancer lines and show similar activity against A549, HeLa and RKOp21 cells.

Millenium Pharmaceuticals claim a series of fused aromatic heterocycles positioned with a pendant hydroxamate as selective inhibitors of HDAC6 for a variety of proliferative disorders, as well as other potential indications [116]. The patent claims a variety of nitrogenous heterocycle-fused phenyl hydroxamic acids with different ring sizes and substitutions in the azacycle portion of the inhibitors. Evaluation against HDAC6 reveals that many compounds in the series show IC₅₀ values between 50 and 100 nM.

HDAC8-selective inhibitors

Recent structural evidence for HDAC8 bound to a variety of inhibitors provided the inspiration for the design of a novel class of hydroxamates from the J David Gladstone Institutes and Ithaca College [117]. The inventors disclose a series of selective 'linker-less' hydroxamic acids that would potentially interact with a unique subpocket in the active site of HDAC8. Moreover, the increased steric bulk of the naphthalene moiety is believed to create a blocking effect that lowers the potency of other HDAC isoforms. This group specifically designed HDAC8 inhibitors that would not interact with HDAC1 and HDAC6. A simple naphthyl hydroxamate (**32a**) is a moderately potent inhibitor of HDAC8 (14 μM) without inhibition of HDAC1 and HDAC6. Insertion of either an olefinic or aryl spacer (**32b** and **32c**, respectively) between the bulky naphthyl unit and the polar headgroup leads to increased HDAC8 inhibition (0.7 and 0.3 μM, respectively), but also moderately increases HDAC6 activity (82 and 52 μM, respectively); however the compounds remain selective against HDAC1.

Selective HDAC8 hydroxamate inhibitors optionally substituted with benzimidazole, indole, azaindole or pyrrole moieties are claimed by Pharmacyclics, Inc. [118]. The inventors focus their efforts on developing selective HDAC8 inhibitors owing to the fact that HDAC8 is expressed at high levels in tumor cell lines including Jurkat, HuT78, K562, PC3 and OVCAR-3. In addition, it has been reported that inhibiting HDAC8 decreases the proliferation of T-cell-derived tumors yet does not affect the growth of nonmalignant cells. The inventors describe the evaluation of several HDAC inhibitors, all of which exhibit IC₅₀ values of less than 1 μM against HDAC8 and at least a tenfold, some with a 100-fold, difference in the inhibition of HDAC1, 2, 3, 6 and 10. The compounds were further evaluated against Jurkat cell lines. The most potent compounds in this assay (including **33** and **34**) show GI₅₀ values of 2.8–5.2 μM. Selected inhibitors induced apoptosis in T-cell lymphoma or leukemia cell lines. Additionally, this compound activates caspases 3, 8 and 9, exhibiting effects on both

the intrinsic and extrinsic apoptotic pathways. Pharmacokinetic parameters such as half-life, volume of distribution and area under the concentration–time curve for compounds **33** and **34** in rats were measured and reported.

Indole-based aryl hydroxamates are claimed by Pharmacyclics Inc., as a general scaffold for the preparation of selective inhibitors of HDAC8 [119]. The inventors show that HDAC8 is expressed in many cancer lines and the knockdown of the enzyme can lead to apoptosis. However, HDAC8 inhibition does not affect the proliferation of normal cells or non-T-cell-derived cancer lines, thus suggesting that selective HDAC8 targeting could be a highly successful strategy. PCI-34051 is toxic against T-cell-derived lines (Jurkat, HuT78; 3–4 μM GI₅₀) but is nontoxic toward B-cell-derived cancers (DB, K562; >20 μM), solid tumor lines and normal human peripheral blood mononuclear cells.

A 2011 disclosure from Methylgene claims a series of hydroxamates as selective inhibitors of HDAC8. The series is based on a hydroxamate, derived from a piperidine substituted with an exocyclic acrylic acid [120]. Several derivatives are reported to inhibit HDAC8 with IC₅₀ values less than 1 μM ; however, no additional data concerning other isozymes were disclosed.

Other groupings

The Kyushu Institute of Technology claim a series of isozyme selective HDAC inhibitors built upon a central cyclic tetrapeptide core containing a pendant thiol group that could be masked in prodrug form by conversion to a disulfide [121]. The disulfides show superior stability in serum; once these compounds were taken into cells they were rapidly reduced to liberate the free thiol. Specific members of this class are shown to potently inhibit HDAC1 and HDAC4 with good selectivity over HDAC6. HDAC6 is reported to be highly expressed in the testes and may be important for differentiation of normal tissue; thus, inhibition of this isozyme may lead to undesired side effects. The inventors studied the free thiol, homodimer disulfide and various mixed disulfides to evaluate HDAC inhibition, cellular growth inhibition and plasma stability. The *S*-pyridyldisulfide derivative **35** is shown to be a potent inhibitor of both HDAC1 and HDAC4, both in the presence and (absence) of dithiothreitol with IC₅₀ values of 0.553 (6.76) and 1.12 (68.3) nM, respectively, with good selectivity over HDAC6 (IC₅₀=1610 nM). Both the free thiol and various disulfide derivatives show good activity in cellular assays, suggesting that the disulfides were readily reduced in the cellular environment. Moreover, compound **35** shows activity against HeLa cells (GI₅₀ = 1.17 μM) with greater than tenfold selectivity relative to a normal cell line (TIG-3). The relative stability of the mixed disulfide **35** was compared with the free thiol and homo-disulfide analogs (**36a** and **36b**, respectively) by incubating in fetal-calf serum and examining residual HDAC inhibitory activity. Both the homodimer (**36a**) and the mixed disulfide **35** are shown to retain their enzymatic inhibitory activity after far longer periods of serum incubation than **36b**, likely a result of protection of the free thiol in the disulfide variants.

An interesting study on related cyclic tetrapeptides by researchers at Riken shows that modification of the zinc-directed side chain can alter isozyme selectivity and that potent HDAC6 inhibition can be realized with this chemotype [122]. The Riken group examined a number of different side-chain modifications ranging from simple capping groups to highly potent metal chelators. The nature of these groups is shown to have an impact on the inhibition profile of the tetrapeptides. Interestingly, a simple trifluoromethylketone functionality gives a compound (**37a**) that potently inhibits HDAC1 (730 nM) and HDAC4 (610 nM), while showing increased selectivity against HDAC6 (1440 nM). The trifluoroketone derivative shows excellent cytotoxicity (~9 nM) against HeLa cells. Furthermore, the application of a more potent metal-binding head group such as that

depicted in compound **37b** yields potent activity for HDAC1 and 4, but with greatly diminished activity against HDAC6 (300 μ M).

A series of hydroxamate-based inhibitors based on a sulfonyl pyrrole scaffold are claimed by 4SC AG (Planegg Martinsrid, Germany) for use against a variety of cancers [123]. An example of this class (resminostat) is now in clinical trials for the treatment of hepatocellular carcinoma. Resminostat inhibits both Class I and II HDACs (1, 3 and 6) potently (42–72 nM) but shows selectivity over HDAC8 (IC_{50} =877 nM). The title compound was shown to be active against several multiple myeloma lines including OPM-2, RPMI-8226 and NCI-H92, with an IC_{50} of approximately 2.5 μ M.

Johns Hopkins University claim a series of alkyl hydroxamate derivatives, such as compound **38** containing a methylene diamine linker fused to a hydrophobic biaryl moiety [124]. At a concentration of 1 μ M, compound **38** inhibits approximately 74% of HDAC activity. Evaluation of potential isozyme selectivity demonstrates modest selectivity against HDAC1. At concentrations that inhibit approximately 90% of the activity of both HDAC4 and HDAC6, the same compound inhibits approximately 60% of HDAC1 activity. The inventors also show that the compound is able to induce apoptosis in a human myeloblastic leukemia cell line (ML-1).

Future perspective

The development of potent inhibitors of histone deacetylases has been a highly successful effort in medicinal chemistry. Although zinc-targeting hydroxymates represent the majority of inhibitor types, several other chemotypes such as 2-aminobenzamides, cyclic peptides and alkyl carboxylates, provide for notable additions. Early work on HDAC inhibitors focused on pan-inhibitors that inhibited most, if not all, of the zinc-dependent members of the family. Vorinostat (SAHA) is the most prominent member of these pan-inhibitors and has been approved for the treatment of lymphoma. HDACs are key players in epigenetic events within the cell as they control the expression level of different genes. It is currently not clear how much of the anticancer activity of HDAC inhibitors stem from epigenetic modulation versus other potential pathways such as tubulin deacetylation. Recent studies suggest that superior agents with increased efficacy and decreased toxicity may be possible if more selective targeting of specific enzymes is achieved. The potential for employing more selective compounds, and hence superior therapeutic agents, has catalyzed a significant amount of new work directed at the discovery and development of isozyme-selective inhibitors. The majority of approaches have relied on many of the previously described classes (hydroxamates, benzamides and peptides) as the point of departure to develop isozyme-selective agents. However, it is anticipated that new chemotypes may be necessary to achieve significant isozyme selectivity. One area that is likely to continue to evolve and have an increasingly important impact is the determination of high-resolution structures of the different HDAC isoforms. Currently, there are four structures of isozymes available for use and it is expected that more will be reported in the future. These novel isozyme structures, as well as new co-crystal structures revealing detailed interactions with various inhibitors, should lead to more rational design in the inhibitors rather than the more random-screening approaches characteristic of many of the reported investigations. A second area that will likely have a major impact on future design efforts will be key biological studies probing the specific targeting of different isozymes, thus suggesting therapeutic advantages that could arise from increased selectivity. As the role of specific isozymes in important disease states becomes clearer, it is likely that the quest for more selective inhibitors will become focused on a few prominent isozymes. Alternatively, it may be that inhibition of specific HDAC isozymes are associated with undesired pharmacological effects. In this case, medicinal chemistry efforts may be more focused on negative design whereby the goal

may be to eliminate binding to a specific enzyme, but allow broad spectrum activity for other family members. Finally, another evolving facet of these studies will be to improve our understanding of the role of HDACs in the regulation of cellular processes and their specific roles in the pathology of disease. Although many of these isozyme selective inhibitors may not become therapeutic agents, they are likely to serve as probe molecules to better dissect the role of individual isozymes in the cell. Arguably, the study of isozyme-selective modulation of HDACs will be one of the most exciting aspects of this area of drug development.

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Executive summary

- Histone deacetylases (HDACs) are a family of enzymes that hydrolyze acetamides derived from the ϵ -amino group of lysine residues in histones and other proteins.
- In histones, removal of capping ϵ -acetamides by HDACs promotes a strong interaction between the positively charged lysine and the negatively charged phosphate groups of DNA, thus preventing transcription of specific genes.
- Inhibition of HDACs is a validated target for the treatment of certain cancers with strong evidence for a role in other diseases.
- The HDACS are a family of at least 18 isozymes that are expressed differentially and are linked to different biological functions.
- Strong evidence suggests that selective targeting of specific isozymes would enhance the therapeutic potential of HDAC inhibitors and lower toxicity.
- A wide range of efforts are being directed toward several different HDAC isoforms.
- Several different classes of HDAC inhibitors are being explored for the development of isozyme-selective inhibitors.
- Recent advances in the structural biology of HDACs is beginning to impact design efforts and helping to rationalize subtle structural differences between isozymes.
- Exciting progress has been made in developing inhibitors with better selectivity profiles than the current approved inhibitors.
- Early evidence supports that increasing selectivity can have a beneficial impact on the therapeutic profile of the inhibitor.

Figure 1. Depictions of structures of HDAC isozymes bound to inhibitors
(A) HDAC2 (salmon) bound to an aminobiphenyl benzamide (dark green), (B) HDAC4 (bright green) bound to a dihydroimidazole hydroxamate (yellow), (C) HDAC7 (purple) bound to SAHA (orange) and (D) HDAC8 (cyan) bound to SAHA (orange). Key active site residues are depicted and the zinc atom is shown as a sphere.

Figure 2.
Small-molecule inhibitors of histone deacetylases.

Table 1

Inhibition of histone deacetylase isozymes.

Compound	Affinity	Selectivity	Ref.
1	Inhibits class 1 enzymes by 97% compared with SAHA	HDAC4/5 85% HDAC3 48% HDAC7 35%	[101]
2	HDAC1 IC ₅₀ = 3.3 nM	HDAC6 > ₅₀ μM	[102]
4-7	Towards Class IIa	Towards Class I	[104]
11	HDAC4 IC ₅₀ = 46 nM HDAC5 IC ₅₀ = 7.2 nM	HDAC1 IC ₅₀ = 0.3 nM HDAC2 IC ₅₀ = 0.4 nM	[105]
12	HDAC4 IC ₅₀ = 6.9 μM HDAC5 IC ₅₀ = 3.2 μM HDAC7 IC ₅₀ = 2.6 μM HDAC9 IC ₅₀ = 9.1 μM	HDAC8 IC ₅₀ = 0.27 μM	[105]
13	HDAC4 IC ₅₀ = 33.1 μM HDAC5 IC ₅₀ = 10.1 μM HDAC7 IC ₅₀ = 11.3 μM HDAC9 IC ₅₀ = 34.1 μM	HDAC8 IC ₅₀ = 0.76 μM	[105]
14	% activity at 0.5 μM HDAC1 = 56.3 HDAC2 = 71.5 HDAC3 = 53.6 HDAC8 = 36.2		[106]
15	% activity at 0.5 μM HDAC1 = 0.4 HDAC2 = 1.0	% activity at 0.5 μM HDAC3 = 34.8 HDAC8 = 33	[106]
16	% activity at 0.5 μM HDAC1 = 1.0 HDAC2 = 0.4	% activity at 0.5 μM HDAC3 = 38.5 HDAC8 = 45	[106]
17	% activity at 0.5 μM HDAC1 = 6 HDAC2 = 7	% activity at 0.5 μM HDAC3 = 50.4 HDAC8 = 52	[106]
18	HDAC1/2 IC ₅₀ = 1.03 nM	HDAC8 IC ₅₀ = 544.6 nM HDAC6 IC ₅₀ = 728.7 nM	[107]
19, 20	HDAC1 IC ₅₀ = 22 nM	HDAC2 IC ₅₀ = 1.3 μM HDAC 8 IC ₅₀ = 0.5 μM	[108]
21a	HDAC4 IC ₅₀ = 95 nM HDAC4 IC ₅₀ = 95 nM	HDAC1 IC ₅₀ = 710 nM HDAC6 IC ₅₀ = 350 nM	[109] [109]
21b	Loss of selectivity	Loss of selectivity	[109]
22	HDAC6 IC ₅₀ = 128.9 nM	HDAC1/2/3/8 IC ₅₀ = >950 nM	[110]
23	HDAC6 IC ₅₀ = 76.2 nM	HDAC1/2/3/8 IC ₅₀ = >950 nM	[110]
24	HDAC6 IC ₅₀ = 14 nM	HDAC1 IC ₅₀ = 13.8 μM HDAC8 IC ₅₀ = 814 nM All other isozymes IC ₅₀ = >30 μM	[111] [111] [111]
25	HDAC6 IC ₅₀ = 14 nM	HDAC1 IC ₅₀ = 5.18 μM	[111]
26	HDAC6 IC ₅₀ = 45 nM	HDAC1 IC ₅₀ = 240 nM HDAC2 IC ₅₀ = 150 nM HDAC3 IC ₅₀ = 390 nM HDAC8 IC ₅₀ = 5000 nM	[112] [112] [112] [112]
27	HDAC6 IC ₅₀ = 4 nM	All other isozymes IC ₅₀ = 1000-fold higher	[113]

Compound	Affinity	Selectivity	Ref.
28–30	HDAC6 IC ₅₀ = 4.4–75 nM	All other isozymes IC ₅₀ = 245–5333 nM	[114]
31	HDAC6 pIC ₅₀ = 5.03	HDAC8 pIC ₅₀ = 7.26	
32a	HDAC8 IC ₅₀ = 14 μM	HDAC1/6 no inhibition	[117]
32b	HDAC8 IC ₅₀ = 0.7 μM	HDAC6 IC ₅₀ = 82 μM HDAC1 no inhibition	[117] [117]
32c	HDAC8 IC ₅₀ = 0.3 μM	HDAC6 IC ₅₀ = 52 μM HDAC1 no inhibition	[117] [117]
33, 34	HDAC8 IC ₅₀ = <1 μM	HDAC1/2/3/6/10 IC ₅₀ = 100–1000-fold greater	[118]
35	HDAC1 IC ₅₀ = 0.553 nM HDAC4 IC ₅₀ = 1.12 nM	HDAC6 IC ₅₀ = 2010 nM	[121]
37a	HDAC1 IC ₅₀ = 730 nM HDAC4 IC ₅₀ = 610 nM	HDAC6 IC ₅₀ = 1440 nM	[122]
37b	Loss of selectivity	Loss of selectivity	[122]
38	Inhibits HDAC4/6	1.3-fold loss in activity for HDAC1	[124]