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Thirty years of HDAC inhibitors: 2020 hindsight

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KEYWORDS. enzyme inhibitors, metalloenzymes, amidohydrolases, epigenetics,
histone deacetylases

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4 ABSTRACT. It is now thirty years since the first report of a potent zinc-dependent
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7 histone deacetylase (HDAC) inhibitor appeared. Since then, five HDAC inhibitors have
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10 received regulatory approval for cancer chemotherapy, while many others are in clinical
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13 development for oncology as well as other therapeutic indications. This perspective
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16 reviews the biological and medicinal chemistry advances over the last three decades
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19 with an emphasis on the design of selective inhibitors that discriminate between the
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24 eleven human HDAC isoforms.
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33 Introduction

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40 The human genome contains a modest number of 20,000-25,000 genes coded by only
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43 5% of its DNA sequence. However, it is not the case that 'one gene = one protein', as
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46 processes such as mRNA editing lead to a transcriptome that is several-fold higher than
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49 the number of genes. A further expansion occurs after protein synthesis through the
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53 post-translational modification (PTM) of amino acid side chains. The majority of PTMs
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3 are dynamic in nature, enabling a single protein to exist in multiple functional states and
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7 move from one state to another in a reversible manner. The ϵ -amino side chain of
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10 lysine, in particular, is the conduit for a plethora of PTMs (Figure 1) via alkylation or
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13 acylation reactions.^{1,2} Alkylation increases the size of the lysine side chain while
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17 preserving its positively charged nature at physiological pH, whereas acylation results in
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20 neutral or even negatively charged side chains ranging from the addition of a single
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23 carbon in formylation to the conjugation of proteins such as ubiquitin. Two of these
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27 processes, acetylation and methylation, occur in the lysine-rich N-terminal tails of
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30 histone proteins where they play a central role in the epigenetic regulation of chromatin
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33 structure and the recruitment of its binding partners to modulate gene transcription.
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38 Consequently, the enzymes involved in lysine acetylation and methylation and their
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41 removal have become important targets for small molecule drug discovery.³ At the
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44 present time, five inhibitors of lysine deacetylation, and one of lysine methylation, have
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48 received regulatory approval as anticancer agents. This year marks the 30th
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51 anniversary of the disclosure of the natural product trichostatin A as the first potent
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55 inhibitor of histone deacetylation. In this perspective, we summarize the progress since
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3 then regarding the biology of lysine deacetylase enzymes, the compounds currently in
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7 clinical development against this target and the discovery of isoform selective inhibitors.
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10 Previous reviews in this journal provide a snapshot of the state of the art at earlier time
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14 points.^{4,5}
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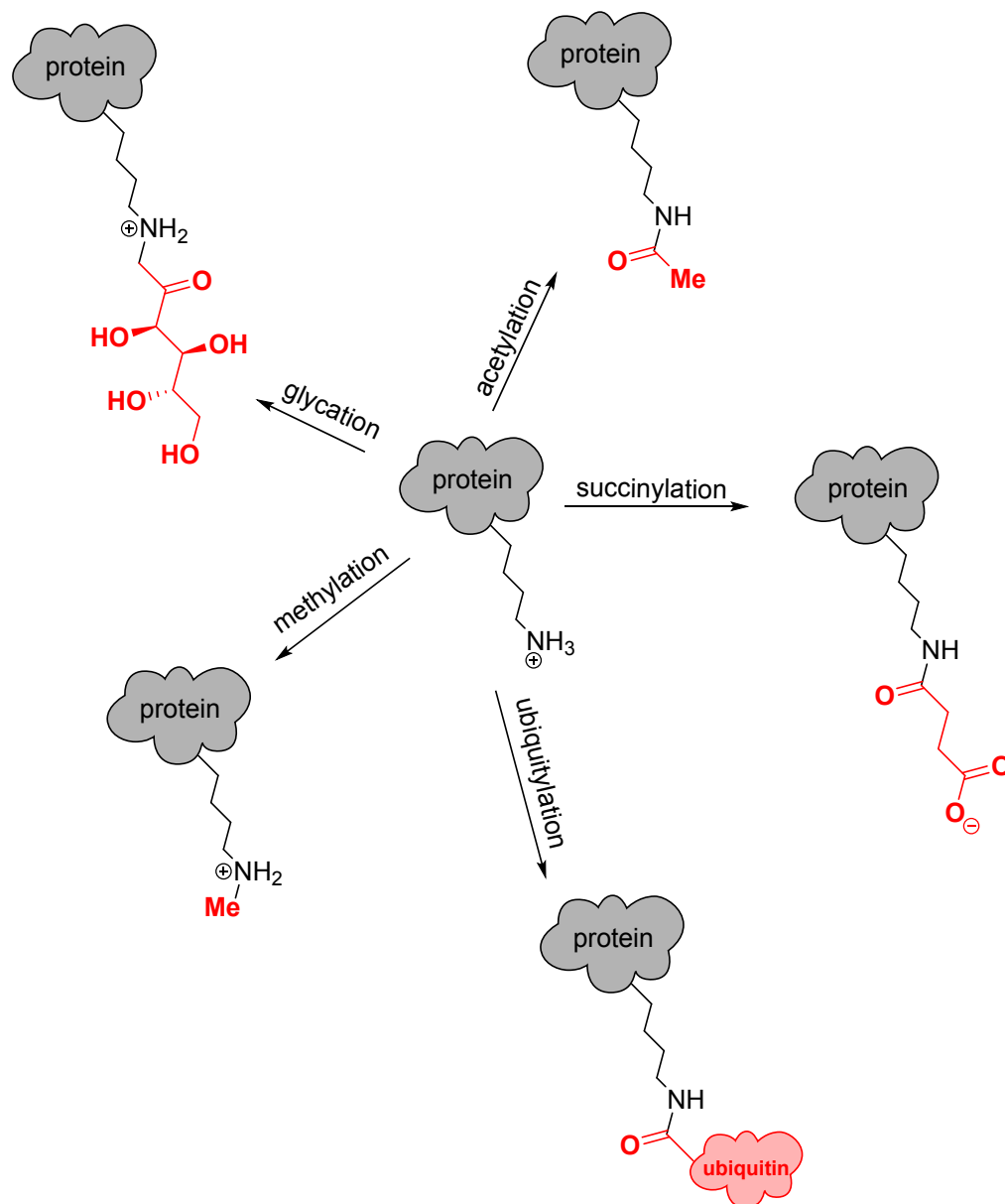


Figure 1. Examples of lysine post-translational modification in proteins.

Lysine acetylation was first observed in the early 1960s in histones due to the abundance of the PTM in these proteins. Soon thereafter, enzymes that catalyze the

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3 forward acetylation reaction and the reverse deacetylation reaction were discovered and
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7 logically named histone acetyltransferases (HATs) and histone deacetylases (HDACs)
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10 respectively. Unfortunately, this historical nomenclature is well entrenched despite its
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13 inaccuracy, as we now know that lysine acetylation is not restricted to histones but a
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17 widespread PTM in thousands of human proteins present in diverse cellular
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21 compartments.⁶ The PTM is produced via the transfer of an acetyl group from acetyl
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24 CoA to a lysine residue, either by a reaction catalyzed by HATs or through an enzyme-
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27 free process. Acetylation can dramatically alter the biological properties of a protein
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31 (Table 1) as well as being the chemical signal for recognition by bromodomains, a
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34 protein-protein interaction subunit found in transcriptional activators.⁷ Meanwhile, a
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38 variety of other acyl CoA species undergo a similar reaction with lysine residues in vivo
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42 to create distinct PTMs.⁸ These include short and medium-chain saturated carboxylic
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45 acids such as formic, propionic, butyric and myristic; short-chain dicarboxylic acids such
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48 as malonic, succinic and glutaric; and carboxylic acids with additional functionality such
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51 as 2-hydroxyisobutyric, crotonic, phosphoglyceric, lipoic and biotin. Although the precise
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55 physiological roles of many of these recently discovered acyllysine PTMs are yet to be
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deciphered, the inadequacy of the nomenclature's emphasis on histone proteins and acetylation is apparent.

Acetylation consequence	Examples
Reduced nucleic acid binding	Histone-DNA, E2F-DNA
Increased protein binding	Histone-PCAF, Histone-BRD4
Reduced protein binding	Hsp90-GR, Ku70-p23
Increased stability	p53, α -tubulin
Decreased stability	DNMT1, HIF1 α
Reduced enzymatic activity	Aurora B kinase, RNase II
Altered cellular localization	STAT3, c-Abl
Altered aggregation	Tau, GRP78

Table 1. Examples of the consequences of lysine acetylation in human proteins.

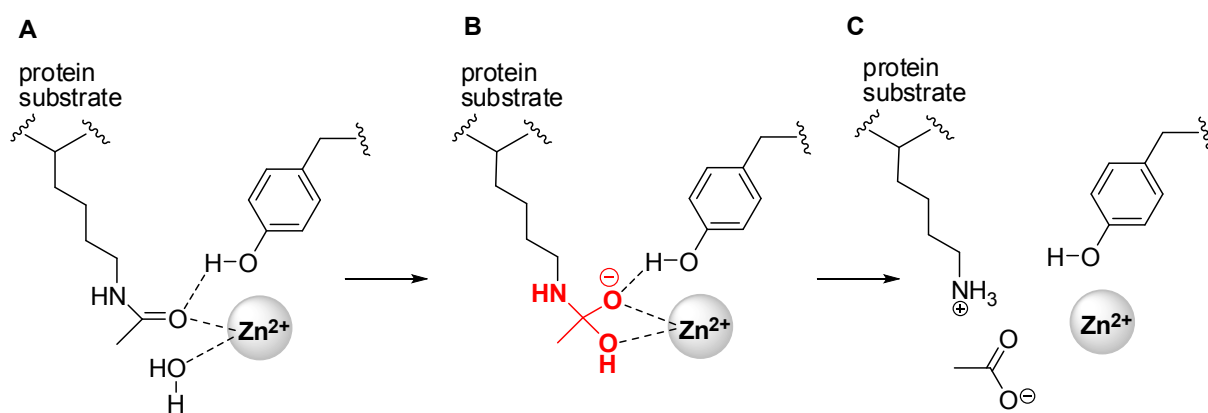
The eleven human HDACs

The reversal of lysine acetylation is accomplished by enzymatic cleavage catalyzed by the HDACs. In humans, there are 18 HDACs that fall into two families based on their catalytic mechanism.⁹ Eleven of the HDACs are zinc-dependent metalloenzymes named HDAC1-11 that hydrolyze the amide bond using water as a nucleophile. The remaining seven sirtuins 1-7 employ NAD⁺ as a cofactor and transfer the acyl group to

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3 the C2 position of the ribose sugar.¹⁰ Although both enzyme families perform the same
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7 chemical reaction of acyllysine cleavage, the term HDAC usually refers to the zinc-
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10 dependent enzymes that are the focus of this perspective.¹¹
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14 X-ray crystal structures are available for the majority of HDAC isoforms with the free
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18 protein, protein-inhibitor complexes as well as substrates bound to mutated proteins that
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22 do not turnover. These extensive studies have provided a detailed understanding of the
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25 catalytic mechanism, as exemplified in the model for HDAC8.¹² The acetyllysine
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29 substrate sits in a narrow channel lined with hydrophobic residues, with a tyrosine
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33 residue flipping in conformation to enable hydrogen bonding with the carbonyl oxygen
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36 (Figure 2A). The hydrophobic nature of the channel, and the conformational change by
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39 the tyrosine residue, serve to provide enzyme specificity for the acyllysine side chain. A
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43 zinc cation, coordinated to aspartate and histidine residues, sits at the end of the
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47 substrate channel, bound to a water molecule activated by a charge relay mechanism
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50 with two adjacent histidine residues. Nucleophilic attack of the carbonyl by the water
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53 produces a tetrahedral oxyanion intermediate (Figure 2B), which collapses to give rise
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3 to the free lysine and acetate products (Figure 2C). In some HDAC isoforms, there is an
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7 additional hydrophobic internal cavity lateral to the active site also known as the 'foot
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10 pocket'. This cavity accommodates acyl side chains longer than acetyl in the case of
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14 HDAC8 and HDAC11 (see below) and may aid the entry of water and the release of
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18 acetate.



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39 **Figure 2.** A simplified illustration of the catalytic mechanism of deacetylation by HDACs:
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41 **A.** Enzyme-substrate complex, with binding interactions between the acetyllysine
42 bearing protein substrate and a tyrosine residue and the zinc cation in the HDAC active
43 site. **B.** The tetrahedral oxyanion enzyme intermediate arising from nucleophilic attack
44 by water. **C.** Enzyme-product complex, with the lysine residue and acetate prior to
45 release from the active site.
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54 It is worth highlighting a few key points regarding the HDAC enzymes:
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4 1. Substrate binding is predominantly through the acetyllysine side chain entering the
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7 hydrophobic channel rather than recognition of the protein backbone. In some cases,
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10 bonding interactions are observed between HDACs and the amino acid residues that
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13 are +1 or -1 with respect to the scissile acetyllysine. However, extended contacts such
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16 as occur in peptide backbone recognizing proteases are rare in HDACs which are
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19 relatively nonspecific in their substrate sequence preferences. The compact nature of
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22 substrate binding is an advantage for medicinal chemistry, as small molecule inhibitors
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28 need only simulate acetyllysine rather than a longer peptide sequence.
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32 2. Coordination between acetyllysine and the active site zinc cation is critical for
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35 substrate binding and catalysis. Indeed, all high affinity HDAC inhibitors barring covalent
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38 modifiers achieve their potency by zinc coordination, acting as either monodentate or
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41 bidentate ligands to mimic the tetrahedral oxyanion intermediate.
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47 3. In broad terms, the features of the HDAC8 catalytic mechanism hold true for other
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50 isoforms. Nevertheless, there are significant variations in the geometry of the active site,
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53 the nature of the catalytic residues, the substrate channel, presence or absence of the
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3 internal cavity and positioning of adjacent protein loops. Selective inhibitors that
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7 discriminate between HDAC isoforms will need to take advantage of such differences.
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11 The human histone deacetylases are further subdivided into four classes according to
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13 their sequence homology and cellular localization: Class I = HDAC1, HDAC2, HDAC3
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15 and HDAC8; Class IIa = HDAC4, HDAC5, HDAC7 and HDAC9; Class IIb = HDAC6 and
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17 HDAC10; Class III = sirtuins 1-7; Class IV = HDAC11. However, this classification is
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19 somewhat arbitrary and masks the similarities or differences between isoforms in terms
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21 of structure and function. In cell-free biochemical assays, HDAC1, HDAC2, HDAC3 and
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23 HDAC6 have high enzymatic activity against simple acetyllysine containing peptides
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25 and will be discussed first, followed by the other isoforms that are more mysterious in
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27 their substrate preferences.
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44 *HDAC1, HDAC2*

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48 These isoforms are core HDACs that are co-expressed and are ubiquitous within and
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50 between species.¹³ Although they efficiently hydrolyze acetyllysine peptide substrates
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52 on their own in vitro, they predominantly exist in the nucleus as homo- and heterodimer
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3 constituents of the NuRD, Sin3A, CoREST, MiDAC and MIER transcriptional repressor
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7 complexes. While there is >80% homology between HDAC1 and HDAC2, they perform
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10 independent roles in development and embryonic knockout of either isoform is lethal.
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14 On the other hand, in conditional knockouts, there is a level of redundancy with
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17 knockout of both isoforms usually needed for a phenotypic effect. HDAC1 and HDAC2
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20 are truly HDACs in the sense that they play a major role in histone deacetylation. In
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24 addition, they deacetylate many other nuclear proteins involved in transcriptional
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28 regulation including p53, E2F, ATM kinase and CAF1. There is crosstalk with other
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32 epigenetic processes such as methylation, as both DNMT1 and LSD1 are among their
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35 substrates.
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39 *HDAC3*

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43 HDAC3 shares ~50% sequence homology with HDAC1 and HDAC2. The divergence is
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47 mainly in the C-terminal region, with HDAC3 containing a domain for binding to the
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51 nuclear NCoR/SMRT complexes.¹⁴ In fact, HDAC3 is unstable without the presence of
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55 either NCoR or SMRT, and their association is also necessary for the activation of
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3 catalysis. Interestingly, the X-ray crystal structure of HDAC3 with the deacetylase-
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7 activating domain of SMRT contained a molecule of inositol tetrphosphate at the
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10 interface, bringing the two proteins together. Like HDAC1/HDAC2, HDAC3 is
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13 ubiquitously expressed and involved in the deacetylation of histones, while other
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17 nuclear protein substrates include STAT3 and FOXP3. In addition to distinct roles in
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20 embryonic development, HDAC3 is implicated in a number of physiological processes
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24 including circadian rhythms, energy metabolism, neuronal function and bone
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27 remodeling.
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31 *HDAC6*

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37 If HDAC1-3 are the nuclear workhorses of lysine deacetylation, then HDAC6 is the
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40 cytoplasmic equivalent.¹⁵ HDAC6 is the largest in size among the HDACs, with a C-
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44 terminal ubiquitin-binding zinc finger domain. A nuclear localization sequence (NLS) is
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47 opposed by a nuclear export sequence (NES) and Ser-Glu-containing tetrapeptide
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50 (SE14) repeats that result in the protein mainly being in the cytoplasm. Through the
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54 deacetylation of α -tubulin and cortactin aided by an N-terminal microtubule-binding
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3 domain, HDAC6 directly impacts upon the cytoskeleton and cell mobility. Moreover,
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7 HDAC6 is involved in the repair of protein misfolding, as deacetylation of Hsp90
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10 restores its chaperone activity while HDAC6's ubiquitin-binding domain fosters binding
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13 to misfolded proteins and their targeting for destruction by aggresomes. Additional
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17 cytoplasmic substrates include Ku70, whose deacetylation leads to a loss in binding to
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20 BAX and thereby inhibition of apoptosis, and Tau, in which deacetylation alters the
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23 propensity for aggregation. Further to these cytoplasmic roles, HDAC6 can also localize
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27 in the nucleus where it functions as a transcriptional repressor similar to HDAC1-3.
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32 Uniquely among the HDACs, HDAC6 contains two catalytic domains CD1 and CD2.
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36 While CD2 is a robust lysine deacetylase in vitro, CD1 was considered inactive for a
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39 long time. However, recent studies reveal that CD1 is a competent deacetylase with the
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42 right substrates, expressing a preference for C-terminal acetyllysine sequences aided
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45 by the presence of a lysine residue in CD1 that forms electrostatic interactions with the
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48 carboxylate.^{16,17} Another unusual feature of HDAC6, unlike other isoforms, is that
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3 knockout mice are viable and healthy, and do not express an obviously defective
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7 phenotype.
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11 *HDAC8*

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16 HDAC8 is the only isoform for which the gene lies in the X chromosome. Although it is
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19 grouped as a Class I nuclear HDAC, it is fundamentally distinct from the HDAC1-3
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22 isoforms in this Class.¹⁸ Unlike the others, HDAC8 is smaller in size and acts
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25 independently, as it is bereft of C-terminal protein-protein interaction domains that
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28 promote incorporation into multiprotein complexes. In vitro, HDAC8 has a lower k_{cat}/K_m
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31 value for the hydrolysis of acetyllysine containing peptides compared to HDAC1-3 or
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34 HDAC6. Nevertheless, the catalytic activity is significantly higher against tetrameric
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37 histone H3/H4 proteins suggesting that histones are, to some extent, in vivo substrates
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40 for HDAC8.¹⁹ Additional nuclear substrates have been identified such as SMC3, p53,
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ERR α and ARID1a. Recently, Lin demonstrated that HDAC8 hydrolyzes acyllysine
peptides with acyl chains of 2-16 carbons, and k_{cat}/K_m for the longer acyl chains of
octanoyl-, dodecanoyl-, and myristoyllysine was higher than for acetyllysine.²⁰ These

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3 longer chain acyl groups are likely to be accommodated by extending beyond the active
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7 site zinc cation to occupy the hydrophobic internal cavity lateral to the substrate
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10 channel. It may be that fatty acid deacylation is a more important physiological function
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13 of HDAC8 than deacetylation, which would explain the relatively small number of known
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16 validated HDAC8 substrates. Physiologically, the incorporation of these longer acyl
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19 chains into lysine PTMs could act as a sensor of energy metabolism by reporting on the
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22 relative abundance of different acyl CoA species. Alternatively, some fatty acid acylation
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25 may occur adventitiously in which case HDAC8 and HDAC11 (see below) could be
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28 operating as proofreading repair enzymes to remove these PTMs.
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35 *HDAC11*

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40 HDAC11 was first reported only in 2002 and is the smallest of the HDAC isoforms, with
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43 its catalytic domain accounting for >80% of the protein sequence. Primarily localized in
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46 the nucleus, it functions as a transcriptional regulator with an important role in
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49 immunomodulation.²¹ As an enzyme, HDAC11 has weak activity against acetyllysine
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54 substrates. Instead, like HDAC8, it efficiently hydrolyzes acyllysine residues with longer
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3 chain lengths such as the C₁₂ dodecanoyllysine and the C₁₄ myristoyllysine.^{22,23}

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7 Although an X-ray structure of HDAC11 is unavailable, homology modeling suggests
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10 long chain acyl groups are accommodated within an internal cavity in the same manner
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13 as HDAC8. Interestingly, HDAC11 is susceptible to product inhibition by free fatty acids
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16 such as myristic, palmitic, and stearic acids while palmitoyl-coenzyme A and myristoyl-
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19 coenzyme A serve to enhance the fatty acid deacylase activity.
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25 *HDAC4, HDAC5, HDAC7 and HDAC9*

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29 These so-called Class IIa isoforms, unlike other HDACs, are expressed in a tissue
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32 specific manner. They are relatively large in size, with an N-terminal domain that binds
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35 to the MEF2 family of transcriptional repressors.²⁴ Both an NLS and NES are present,
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38 enabling the Class IIa isoforms to move between the nucleus and cytoplasm. The
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41 shuttling is regulated by the reversible phosphorylation of multiple serine residues, with
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44 the unphosphorylated form being nuclear and acting to influence transcriptional
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47 regulation. The C-terminal region contains the active site in which the key tyrosine
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51 residue (Figure 2) is replaced by a histidine, resulting in weak catalytic activity. By using
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3 more electrophilic trifluoroacetyllysine containing peptides, it is possible to carry out in
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7 vitro assays and profile HDAC inhibitors against these isoforms. Whether they are truly
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10 enzymes in vivo or serve to recognize and bind acetyllysine containing proteins is
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13 unclear. The evidence suggests they act as a scaffolding protein, bringing together the
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16 acyllysine substrate and HDAC3 containing multiprotein complexes, with the actual
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19 hydrolysis carried out by HDAC3. On the other hand, it is worth noting that the
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22 archetypal zinc amidohydrolase thermolysin contains a histidine residue in a similar
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25 location within the active site that participates in catalysis.²⁵ Thus, it is conceivable that
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28 the Class IIa isoforms are catalytically competent, and their true substrates have yet to
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31 be identified. Alternatively, these HDACs may perform dual functions of recognition of
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34 an acetyllysine signal followed by slow catalysis as a means of eventual signal
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37 termination, in a similar fashion to the binding of GTP to GTPases which then destroy
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45 the nucleotide.
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50 *HDAC10*
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3 HDAC10 is found in both the nucleus and cytoplasm, and involved in processes such as
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7 autophagy, immunoregulation and DNA repair.²⁶ While it appears to function as a
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10 transcriptional repressor, this activity was puzzlingly independent of lysine
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13 deacetylation. Recent studies from Christianson have clarified this anomaly and
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17 convincingly demonstrated that HDAC10 is an acetylpolyamine hydrolase evolutionarily
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20 related to the prokaryotic homologue.^{27,28} In HDAC10, a unique 3₁₀ helix defined by the
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23 P(E,A)CE motif serves to narrow the hydrophobic substrate channel, leading to
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27 selectivity for acetylpolyamines over more bulky peptides or proteins containing
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30 acetyllysine residues. While the HDAC10 active site contains the conserved tyrosine
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33 and histidine residues and the zinc activated water typical in HDAC catalysis (Figure 2),
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37 an additional negatively charged glutamate residue unique to this isoform acts as a
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41 gatekeeper to favor the binding of cationic acetylpolyamine residues. In fact, the
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45 substitution of this glutamate residue by leucine was sufficient to restore acetyllysine
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49 hydrolytic activity in the mutant enzyme.
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53 *Overview*

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3 Our understanding of HDACs is continually evolving with new insights regarding their
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6 substrates. Until recently, it seemed the family could be unified under the nomenclature
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10 of protein lysine deacylases or KDACs that would encompass both deacetylation as well
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13 as the hydrolysis of other amides. However, the discovery that HDAC10 is a small
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16 molecule polyamine deacetylase means that even the KDAC terminology is no longer
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19 accurate. Meanwhile, whether HDAC4, HDAC5, HDAC7 or HDAC9 are truly enzymes is
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22 debatable. The traditional subdivision of human HDACs according to Class I-IV does
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25 not take these recent developments into account, and we prefer to group them
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28 according to their in vitro substrate tolerance (Figure 3, Table 2).
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36 While this perspective is focused on the human HDACs, homologues in other species
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39 offer additional opportunities for drug discovery. The selective targeting of fungal
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42 HDACs as a means to create novel antifungal agents is one example.²⁹ The
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45 combination of fluconazole and MethylGene's MGCD209, an inhibitor of the fungal
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48 Hos2 HDAC,³⁰ reached Phase 2 clinical trials for the treatment of severe vulvovaginal
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51 candidiasis, but unfortunately did not show improved efficacy over fluconazole alone.
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Another major area of research is the HDACs present in parasites responsible for neglected tropical disease. The approved HDAC inhibitors and clinical candidates as well as novel compounds intended to selectively inhibit the parasite enzymes have shown promising results in in vivo models, as summarized in a recent review.³¹

<i>In vitro Acetyl-Lys peptide hydrolases</i>			
Isoform	Major localization	Key features	Substrate examples
HDAC1, HDAC2	Nucleus	Exist in vivo as NuRD, Sin3A, CoREST, MiDAC and MIER complexes	Histones, transcriptional regulators
HDAC3	Nucleus	Complexation with NCoR/SMRT needed for catalysis	Histones, transcriptional regulators
HDAC6	Cytoplasm	Ubiquitin and microtubule binding domains, two catalytic domains	Tubulin, cortactin, Hsp90, Tau
<i>In vitro longer chain Acyl-Lys peptide hydrolases</i>			
HDAC8	Nucleus	Moderate AcLys hydrolysis with extended sequence	Histones, p53, SMC3, longer chain

		recognition, superior for C ₈ , C ₁₂ , C ₁₄ acyllysine	acyllysine residues
HDAC11	Nucleus	Preference for C ₁₂ , C ₁₄ acyllysine	longer chain acyllysine residues
<i>In vitro Ac-polyamine hydrolase</i>			
HDAC10	Cytoplasm	Narrow substrate channel with glutamate gatekeeper	Acetylpolyamines
<i>In vitro trifluoroAc-Lys peptide hydrolases</i>			
HDAC4, HDAC5, HDAC7, HDAC9	Nucleus and cytoplasm	Tissue-specific, MEF2 binding domain	None? Recruit substrates for HDAC3

Table 2. The eleven human HDAC isoforms summarized according to substrate preference, localization, and key features.

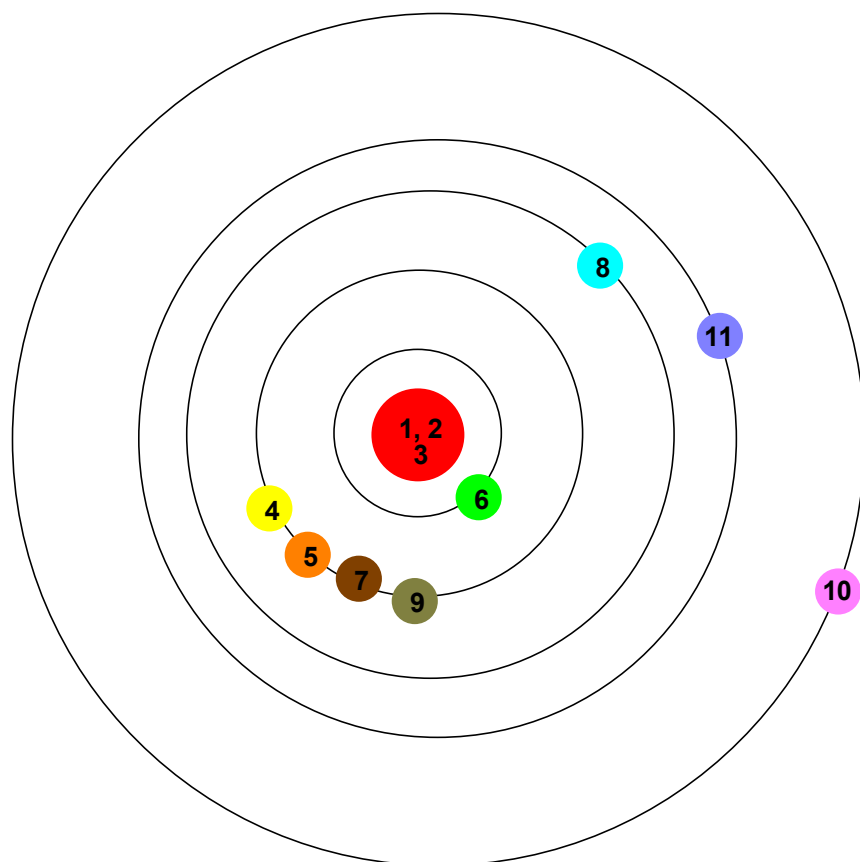


Figure 3. A 'solar system' of human HDACs: at the core are HDAC1, HDAC2 and HDAC3 which exist in multiprotein complexes in the nucleus. These isoforms deacetylate histones and transcriptional regulators, and homologues are ubiquitous across life forms. Next is HDAC6, responsible for the deacetylation of cytoplasmic proteins, followed by an 'asteroid belt' of the pseudoenzymes HDAC4, HDAC5, HDAC7 and HDAC9 with poor catalytic activity. The 'outer planets' HDAC8 and HDAC11 preferentially hydrolyze non-acetyl acyllysine substrates while the distant HDAC10 is a polyamine deacetylase.

Profiling HDAC inhibition

The in vitro profiling of HDAC inhibitors in cell-free enzymatic assays invariably involves the monitoring of product formation using acetyllysine derivatives or peptides based on the N-terminal histone tail sequence that contain an acetyllysine residue.³² Assay kits often employ peptides with an AcLys-AMC (aminomethylcoumarin) residue at the C-terminus which undergoes acetyllysine hydrolysis by HDACs (Figure 4). The resulting C-terminal lysine is recognized by the exopeptidase trypsin, which cleaves the backbone amide to release free aminomethylcoumarin that fluoresces at a longer wavelength than the substrate. Although less amenable to high-throughput screening, direct detection of product formation by chromatographic separation or mass spectrometry without the need for trypsin cleavage are among the alternative formats.³³

In the past, crude cell extracts were used as the source of HDACs, and these mainly contained the nuclear isoforms in an unknown ratio. Nowadays, using recombinant HDAC proteins, the detailed profiling of compounds to determine their subtype selectivity against all eleven isoforms can be accomplished.

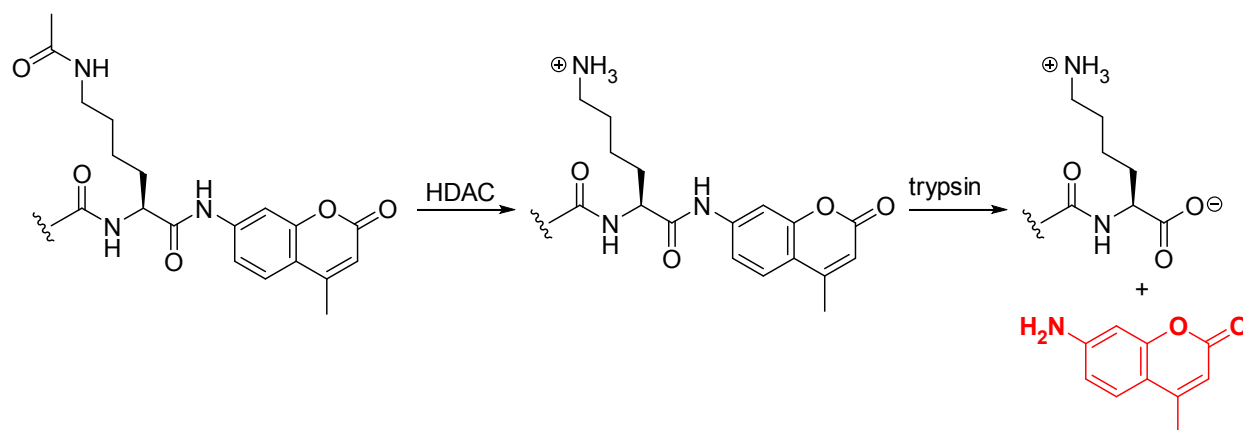


Figure 4. A fluorescence-based assay for measuring HDAC activity based upon product cleavage by trypsin to release aminomethylcoumarin.

Due to differences in protein stability and substrate preference, running HDAC assays against individual isoforms is actually complicated in practice. With regards to the protein, there are options involving full-length enzyme or catalytic domain only, or the addition of binding partners to more closely approximate the cellular environment. Purity, with the possibility of residual contamination by other isoforms, and batch to batch variation are further issues. Simple acetyllsine containing substrates undergo efficient hydrolysis by recombinant HDAC1, HDAC2 and HDAC6 in the fluorescent assay, as well as HDAC3 when co-expressed with NCoR.^{34,35} Although such assays are operationally convenient, it should be kept in mind that the true substrates are proteins

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3 (or multimeric complexes thereof) rather than acetyllysine derivatives or peptides.
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7 Beyond these isoforms, further tinkering is needed for the other HDACs which have
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10 lower catalytic activity for acetyllysine hydrolysis. By switching to more labile (but
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13 artificial) trifluoroacetyllysyl substrates in lieu of acetyllysine derivatives, or using
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17 mutated enzymes with higher turnover, the inhibition can be quantitatively measured.
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21 However, these assays may not accurately reflect the in vivo functions of the enzyme.
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24 For example, HDAC11 preferentially acts upon non-acetyl lysine PTMs and a recent
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27 assay employing a longer acyllysine substrate showed significant differences compared
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30 to the traditional trifluoroacetyllysine-based method.³⁶ Similarly, the current HDAC10 kits
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33 are based on peptides rather than more meaningful acetylpolyamine substrates. As a
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37 result of all these complexities, care should be exercised in the interpretation of
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41 literature HDAC IC_{50} or K_i values taken at different times or from different laboratories,
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45 and the experimental procedures consulted for the exact assay method employed and
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49 the data obtained for reference standards.
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4 In cell-based studies, HDAC inhibitors are usually profiled by their growth inhibition of
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7 human cancer cell lines, with readouts in other cell types used for non-cancer
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10 therapeutic indications. Evidence of HDAC target engagement is commonly
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13 demonstrated by western blotting of the substrate proteins such as histones for nuclear
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15
16 HDACs, p53 for HDAC1 and tubulin for HDAC6, to show a dose-dependent elevation of
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18
19 acetylation levels. Cellular thermal shift assays (CETSA) are a version that allow for the
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21
22 quantification of ligand binding in intact cells.³⁷ The NanoBRET technology based on
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25 competitive binding between inhibitor and a fluorescent tracer is an alternative approach
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28 that enables the measurement of target engagement and drug residence time in cells.³⁸
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34 In addition, downstream effects as a result of HDAC inhibition can be measured,
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37 ranging from phenotypic changes such as cell differentiation to altered protein
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40 expression levels such as induction of the p21 (CIP1/WAF1) cyclin-dependent kinase
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43 inhibitor.
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54 **Natural product HDAC inhibitors**

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3 To inhibit a HDAC, the logical starting point is to mimic the tetrahedral oxyanion enzyme
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6 intermediate (Figure 2B) and design a molecule that coordinates to the active site zinc
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8
9 cation with a slender hydrophobic linker to fit the substrate binding channel. In addition,
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11
12
13 it is helpful to anchor the molecule at the other end with a cap that can potentially
14
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16 engage in additional binding interactions with the rim of the enzyme. In fact, this simple
17
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19 model for a HDAC pharmacophore composed of three elements - zinc binding group,
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22 linker and cap - is sufficiently powerful to account for the vast majority of HDAC
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27 inhibitors and is widely used since it was proposed by Jung in 1997.³⁹
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32 As the medicinal chemist *par excellence*, nature has successfully used the three-point
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34
35 model for the design of nanomolar HDAC inhibitors with a variety of zinc binding groups,
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38 linkers and caps. As mentioned in the introduction, the *Streptomyces* metabolite
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40
41 trichostatin A (1, Figure 5) was the first potent HDAC inhibitor to be identified, by
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45 Yoshida thirty years ago.⁴⁰ Trichostatin A perfectly illustrates the HDAC
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49 pharmacophore: the hydroxamic acid functions as a bidentate zinc chelator, as later
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52 confirmed through X-ray crystallography of trichostatin A-HDAC complexes, while the
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3 diene is a rigid linker that sits in the substrate channel and terminates with a substituted
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7 phenyl ring as the cap. The cap's tertiary amine substituent, protruding out of the
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10 enzyme surface, presumably aids in drug solvation by the aqueous environment.
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14 Psammaplin A (**2**) isolated from a marine sponge is a symmetrical prodrug, undergoing
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17 disulfide reduction to the active molecule which conforms to the three-point
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20 pharmacophore with the thiol as a monodentate zinc binding group.⁴¹ Since thiols have
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23 poor bioavailability, the disulfide protection ensures higher stability and cell permeability
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27 prior to metabolic activation. A masked thiol prodrug features once again in the
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30
31 depsipeptide family of HDAC inhibitors, either as a reducible disulfide in the bacterial
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34 natural products romidepsin (FK228, **3**) and spiruchostatin A (**4**) or as a hydrolytically
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37 labile thioester in largazole (**5**) of cyanobacterial origin.⁴² Upon metabolism, the free
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41 thiol common to all three natural products becomes the zinc binding group, as observed
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45 in the X-ray structure of largazole with HDAC8.
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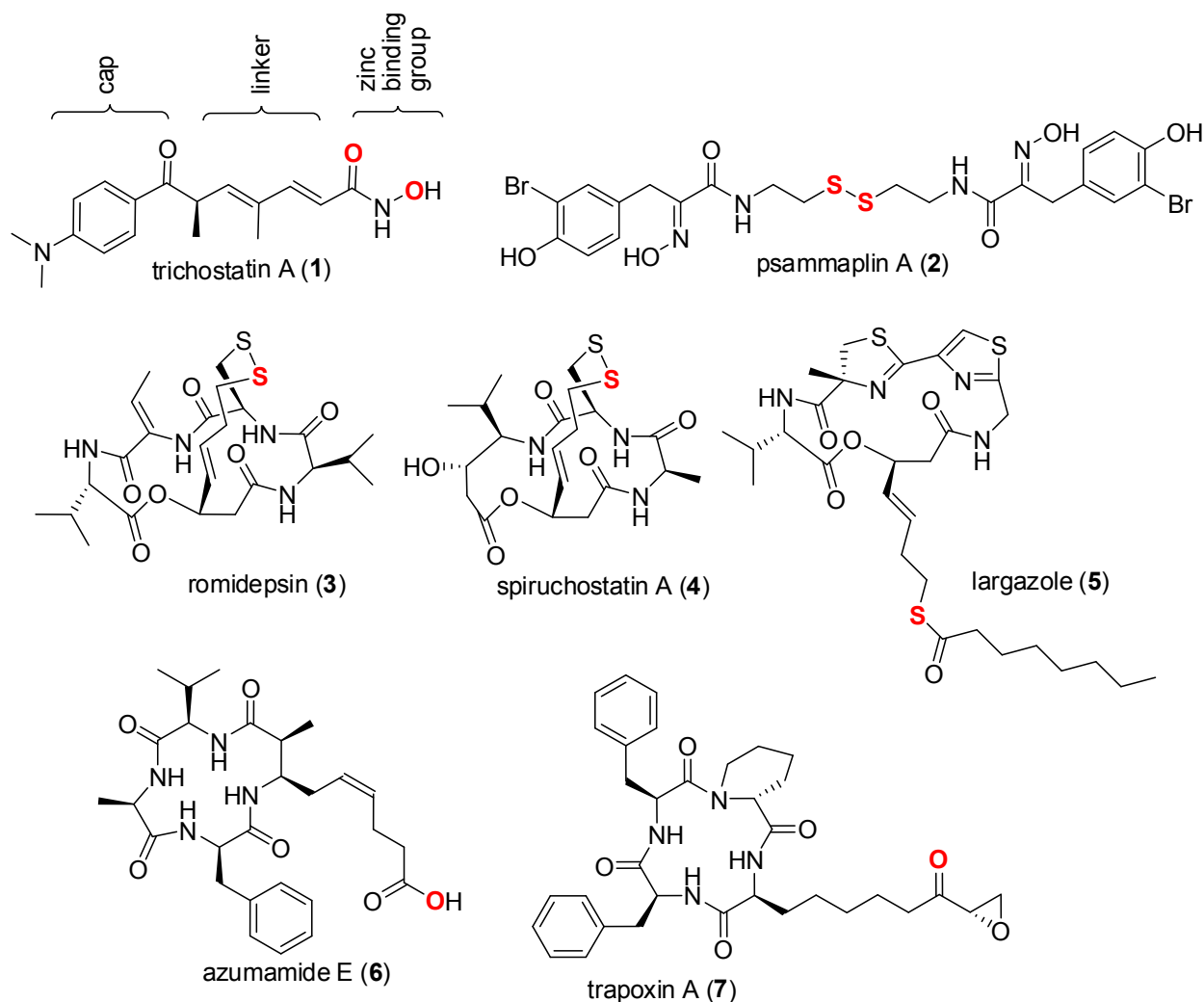


Figure 5. Examples of potent natural product HDAC inhibitors, with zinc binding atoms indicated in red.

While trichostatin A with its simple aromatic cap is a nonselective HDAC inhibitor, both romidepsin and largazole contain larger macrocyclic caps that contribute to their enzyme affinity as well as enable isoform discrimination.⁴³ The importance of

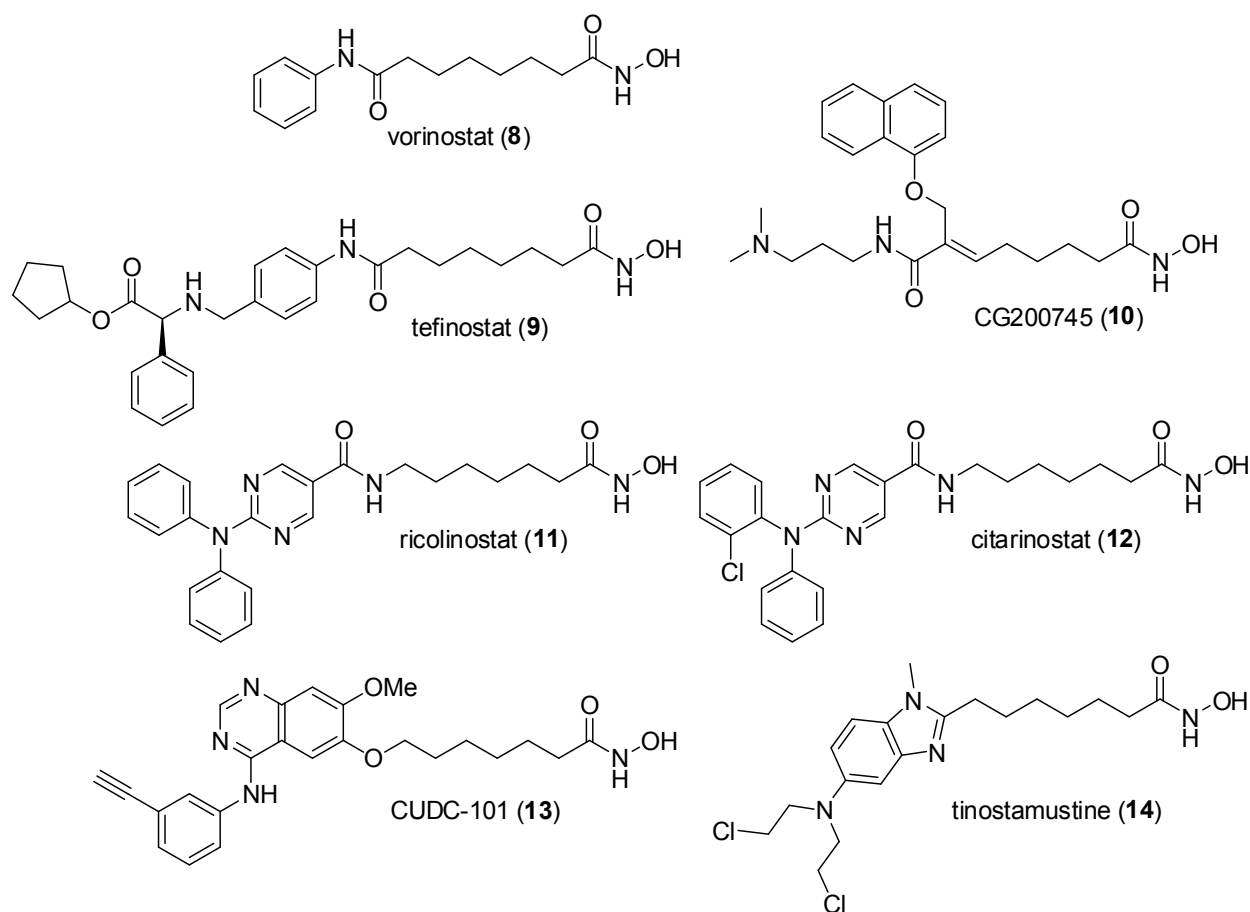
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4 augmenting zinc and substrate channel binding with additional interactions is illustrated
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7 by the marine sponge natural product azumamide E (**6**). Synthetic carboxylic acids have
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10 fared poorly as HDAC inhibitors despite their successful use as zinc binding groups
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13 against other metalloenzymes. Although the short chain butyric, phenylbutyric and
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16 valproic acid have been explored as HDAC inhibitors in the clinic, their sole redeeming
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19 factor is the existence of prior human safety and pharmacokinetic data. The actual
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22 inhibition of HDACs by these carboxylic acids is weak, verging upon the high
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25 micromolar level, and they will not be discussed further. Azumamide E, on the other
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28 hand, achieves submicromolar potency with selectivity for HDAC1 over HDAC6 and the
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31 macrocyclic scaffold presumably makes a significant contribution to these aspects.
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34 Interestingly, a synthetic analogue with the carboxylic acid replaced by a bidentate
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37 hydroxamic acid is even more active as a HDAC inhibitor.⁴⁴ Trapoxin A (**7**), one of a
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40 family of fungal cyclic tetrapeptide HDAC inhibitors, is another interesting example as
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43 ketones are normally inefficient ligands for zinc. The X-ray structure of **7** with HDAC8
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46 indicates the ketone is a substrate mimic, reacting with the active site water to form a
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49 tetrahedral *gem*-diolate which then functions as a bidentate zinc binding group.⁴⁵
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3 Although medicinal chemists work on many epigenetic targets for drug discovery,
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7 HDACs are apparently the only one that nature has taken seriously and the only one
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10 against which highly active secondary metabolites have been discovered. This
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13 emphasis probably reflects the producing microbial or marine organism's need for
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17 chemical defense against competing species or predators. In this regard, the rapid
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20 toxicity arising from HDAC inhibition is evolutionarily more helpful compared to the
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23 slower consequences of the disruption of DNA or histone methylation. A further
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27 question is which HDAC should be inhibited, with the core HDAC1/HDAC2 isoforms
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30 being prime candidates due to their direct impact upon gene transcription and the
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33 existence of homologues in virtually all species. Indeed, it is unlikely to be a coincidence
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37 that all the natural products in Figure 5 strongly inhibit HDAC1/HDAC2 with nanomolar
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40
41 IC_{50} values, and except for the nonselective trichostatin A, are less active against the
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44 cytoplasmic HDAC6. Turner has suggested that evolutionary exposure to natural
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47 product and environmental HDAC inhibitors may have forced organisms to develop
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50 resistance mechanisms, and these same pathways are now subverted by tumor cells to
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55 their advantage upon chemotherapy with such agents.⁴⁶
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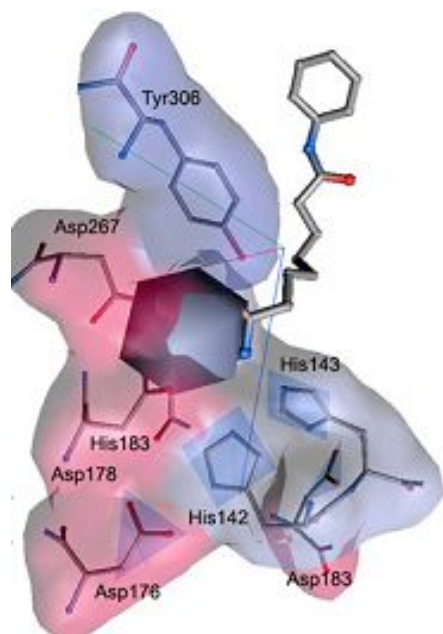
Clinical candidate and approved HDAC inhibitors

In a *tour de force* of chemical biology, Breslow started with DMSO as a lead for cell differentiation and eventually progressed to a more active series of hydroxamic acids culminating in suberoylanilide hydroxamic acid (SAHA).⁴⁷ Later acquired by Merck, SAHA now known as vorinostat (**8**, Figure 6) became the first HDAC inhibitor to receive FDA approval, in 2006 for the treatment of cutaneous T-cell lymphoma. In parallel, the depsipeptide natural product romidepsin (**3**) was also discovered on the basis of phenotypic activity before its molecular target was identified. Romidepsin advanced to clinical development by Gloucester Pharmaceuticals before receiving FDA approval in 2009, for cutaneous T-cell lymphoma as well. Another natural depsipeptide, spiruchostatin A (OBP-801, **4**) is under investigation in Phase 1 clinical trials for the treatment of solid tumors.⁴⁸ Meanwhile, the elucidation of the mechanism of action of trichostatin A led to tremendous medicinal chemistry interest in hydroxamic acids as HDAC inhibitors and they remain the most popular choice of zinc binding group against

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4 this target. Subsequent to vorinostat, six other clinical candidates tefinostat (9),⁴⁹
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7 CG200745 (10),⁵⁰ ricolinostat (11),⁵¹ citarinostat (12),⁵² CUDC-101 (13)⁵³ and
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10 tinostamustine (14)⁵⁴ have appeared that feature similar aliphatic hydroxamic acids with
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14 linear methylene chains. The X-ray structure of the vorinostat-HDAC8 complex (Figure
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18 7) illustrates the typical bidentate coordination observed with hydroxamic acids within
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21 the HDAC active site.⁵⁵



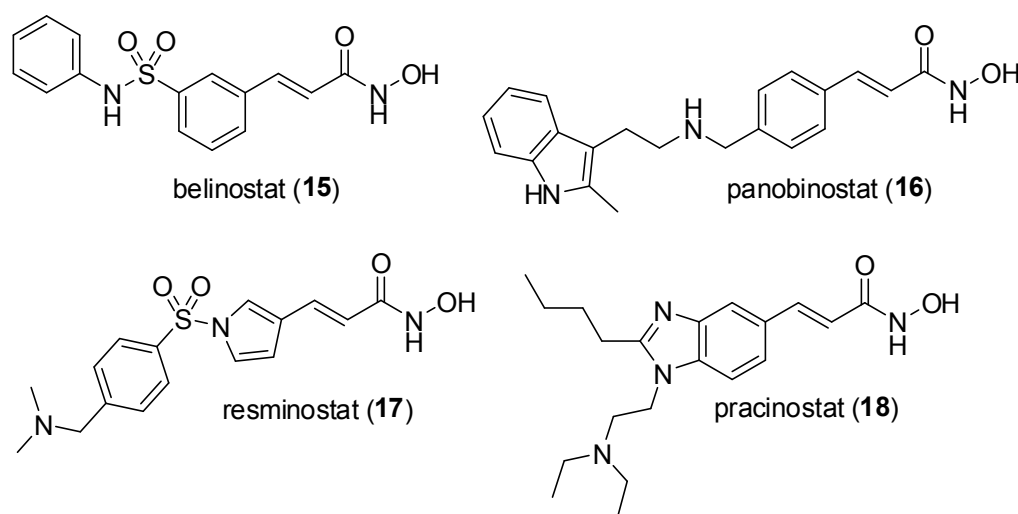
54 **Figure 6.** Clinical candidate HDAC inhibitors with aliphatic hydroxamic acids.



27 **Figure 7.** The binding interactions in the vorinostat-HDAC8 X-ray structure. The zinc
28 cation is displayed as a centroid with the coordinating Asp and His residues in pink. The
29 His and Tyr residues involved in water activation and substrate binding respectively are
30 indicated in blue. Reprinted with permission from ref. 55.
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38 A second family of HDAC inhibitors, inspired by trichostatin A, contains a more rigid
39 alkenyl hydroxamic acid. In 2014, belinostat (**15**, Figure 8) originating from Prolifix and
40 now marketed by Onxeo/Spectrum, became the third HDAC inhibitor to be approved for
41 T-cell lymphoma after vorinostat and romidepsin.⁵⁶ A year later, Novartis's panobinostat
42 (**16**) was approved for the treatment of relapsed or refractory multiple myeloma by both
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4 the FDA and the EMA.⁵⁷ Two other alkenyl hydroxamic acids resminostat (**17**)⁵⁸ and
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6
7 pracinostat (**18**)⁵⁹ are in clinical trials. A newer series of compounds contains an even
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9
10 more rigid phenylhydroxamic acid: givinostat (**19**, Figure 9),⁶⁰ abexinostat (**20**),⁶¹ AR-42
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13 (**21**)⁶² and bisthianostat (**22**).⁶³ A more polar pyrimidinyl heteroaromatic hydroxamic acid
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16 was employed in quisinostat (**23**),⁶⁴ nanatinostat (**24**)⁶⁵ and fimepinostat (**25**).⁶⁶
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41 **Figure 8.** Clinical candidate HDAC inhibitors with alkenyl hydroxamic acids.
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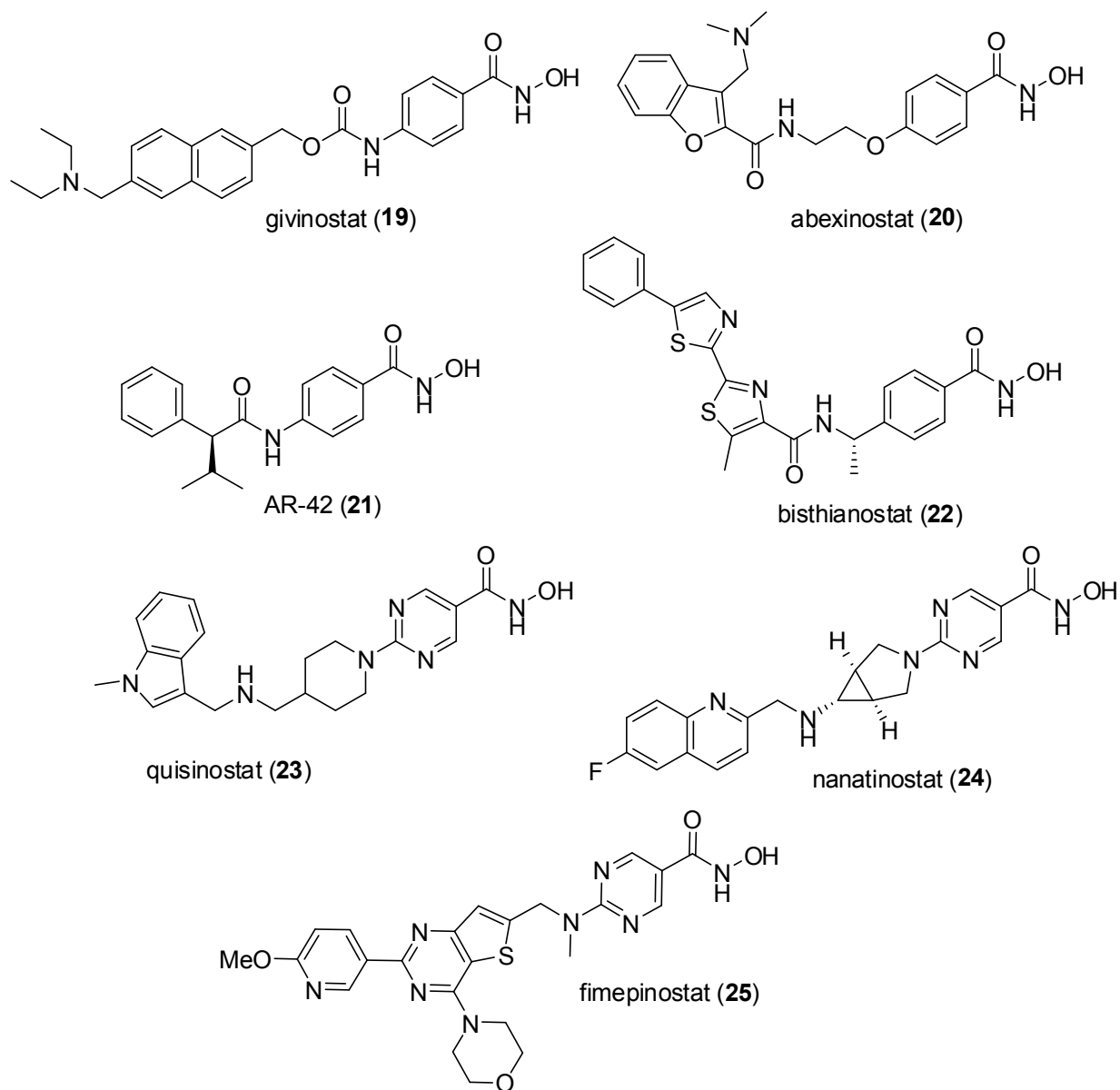


Figure 9. Clinical candidate HDAC inhibitors with aromatic hydroxamic acids.

Pharmacodynamic data against individual HDAC isoforms is available for the approved drugs and the majority of clinical candidates (Table 3). Romidepsin has the typical profile of natural product HDAC inhibitors, with a high activity against the nuclear isoforms HDAC1-3.⁶⁷ There is selectivity, as the natural product is sparing against HDAC8 and HDAC6 although the non-acetyllysine hydrolyzing HDAC10 and HDAC11 are also strongly inhibited. Compared to romidepsin, the forerunner of the hydroxamic acids, vorinostat, has a saturated aliphatic linker and a phenyl cap. With its relatively simple and unconstrained structure, vorinostat would not be predicted to be selective (nor was it intended to be, since the subtleties of isoform function were unknown at the time), and in biochemical enzyme assays it inhibits both the nuclear and cytoplasmic HDACs indiscriminately at submicromolar levels. Although the later hydroxamic acids show an extensive diversity in the architecture of their linker and cap regions, they share a common pharmacological profile insofar that all potently inhibit HDAC1-3 just like vorinostat or romidepsin.

	HDAC isoform IC ₅₀ , nM										
Cpd.	1	2	3	8	4	5	7	9	6	10	11

romidepsin (3)	1	1	1	>100 0	647	>100 0	>100 0	>100 0	22 6	1	0.3
vorinostat (8) ^a	60	42	36	173	20	36	129	49	29	60	31
ricolinostat (11)	58	48	51	100	>1000	>1000	>1000	>1000	5	-	>1000
citarinostat (12)	35	45	46	137	>1000	>1000	>1000	>1000	3	-	-
belinostat (15) ^a	26	22	19	22	15	25	51	24	10	59	27
panobinostat (16) ^a	3	2	2	22	1	1	2	1	1	31	4
pracinostat (18) ^a	28	27	19	48	16	21	104	24	247	23	24
givinostat (19)	133	293	136	837	>1000	532	524	512	312	331	287
abexinostat (20)	21	63	148	370	60	48	350	168	12	52	14
bisthianostat (22)	4	13	6	17	>1000	>1000	>1000	>1000	2	2	78
quisinostat (23)	0.1	0.3	5	4	0.6	4	119	32	77	0.5	0.4
fimepinostat (25)	2	5	2	191	409	674	426	554	27	3	5

Table 3. IC₅₀ values of clinical candidate and approved thiol and hydroxamic acid HDAC inhibitors. ^aFor these compounds, K_i values are given, from a side-by-side comparison in ref. 59. Data for other compounds compiled from refs. 51, 52, 60, 61, 63, 64 and 66.

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3 Besides the hydroxamic acids, *ortho*-aminoanilides (or benzamides) are a second class
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6 of synthetic HDAC inhibitors that have yielded clinical candidates. X-ray crystallography
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9 of enzyme-inhibitor complexes shows bidentate coordination of the carbonyl oxygen
10
11
12 and aniline nitrogen with the active site zinc cation. Nevertheless, *ortho*-aminoanilides
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15 would be expected to be weaker metal binders than hydroxamic acids, and this is
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17
18 reflected in their typical micromolar IC₅₀ values in standard assay conditions compared
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20
21 to the nanomolar potency of the latter. However, the *ortho*-aminoanilides exhibit unusual
22
23
24 kinetics, being tight-binding inhibitors with slow-on/slow-off rates that benefit from pre-
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26
27 incubation and equilibration compared to the fast-on/fast-off behavior observed with
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30 hydroxamic acids. For example, with pre-incubation, an *ortho*-aminoanilide had K_i
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33 values of 148 and 5 nM respectively against HDAC1 and HDAC3, compared to 5 and 8
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35
36 nM respectively for vorinostat (whose activity was unchanged upon pre-incubation).⁶⁸
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38
39 Pfizer's tacedinaline (**26**, Figure 10)⁶⁹ was an early clinical candidate with a simpler cap
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41
42 compared to entinostat (**27**)⁷⁰ and mocetinostat (**28**).⁷¹ Tucidinostat (**29**) from Shenzhen
43
44
45 Chipscreen Biosciences received approval from the CFDA in 2015 for the treatment of
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48 peripheral T-cell lymphoma and marks the first example of a 'Made in China' drug
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3 where the entire drug discovery process was carried out there.⁷² Domatinostat (**30**)⁷³
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7 and CXD101 (**31**)⁷⁴ are additional *ortho*-aminoanilides in clinical trials.
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11 The available literature data on *ortho*-aminoanilides (Table 4) indicates they are similar
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14 as a class, inhibiting HDAC1-3 while poorly active against HDAC6 and HDAC8. The
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17 larger dimensions of the *ortho*-aminoanilide compared to a thiol or hydroxamic acid zinc
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21 binding group can be accommodated by the lateral cavity present in HDAC1-3 but
22
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24 absent in HDAC6. Although HDAC8 contains the lateral cavity, the substitution of a
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27 tryptophan residue for a leucine present in HDAC1-3 leads to a shallower channel that
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31 does not bind to the *ortho*-aminoanilide. To some extent, the *ortho*-aminoanilides are
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35 similar in their activity profile to romidepsin and related depsipeptide natural products in
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39 that they predominantly inhibit HDAC1-3.
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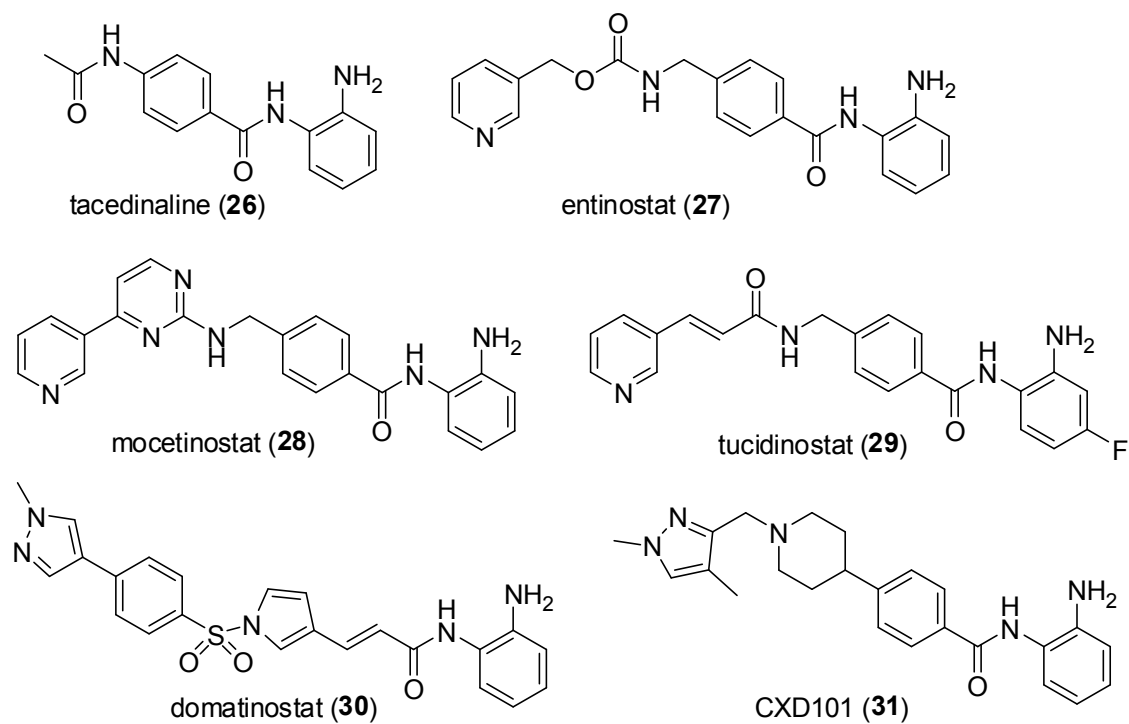


Figure 10. Clinical candidate *ortho*-aminoanilide HDAC inhibitors.

Cpd.	HDAC isoform IC ₅₀ , μM										
	1	2	3	8	4	5	7	9	6	10	11
tacedinaline (26)	0. 9	0. 9	1. 2	>1 0	- 0	- 0	- 0	- 0	- 0	- 0	- 0
entinostat (27)	0. 2	1. 2	2. 3	>1 0	>1 0	- 0	>1 0	0.5 0	>1 0	- 0	- 0
mocetinostat (28)	0. 2	0. 3	1. 7	>1 0	>1 0	>1 0	>1 0	- 0	>1 0	- 0	0. 6
tucidinostat (29)	0. 1	0. 2	0. 1	0.7 0	>1 0	>1 0	>1 0	>1 0	>1 0	0.1 0	0. 4
domatinostat (30)	1. 2	1. 1	0. 6	>1 0	>1 0	>1 0	>1 0	>1 0	>1 0	>1 0	9. 7

CXD101 (31)	0. 1	0. 6	0. 6	-	-	-	-	-	-	-
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Table 4. IC₅₀ values of clinical candidate and approved *ortho*-aminoanilide HDAC inhibitors compiled from refs. 69-74. Note that values are in μM, unlike the nM in Table 3 and assay conditions may not have involved enzyme pre-incubation.

In addition to the compounds discussed above, there are HDAC inhibitors in Phase 1 clinical trials with undisclosed structures: CKD-504 and CKD-506 (Chong Kung Dan Pharmaceutical Corp.), CS3003 (CStone Pharmaceuticals), HG146 (HitGen), KA2507 (Karus Therapeutics) and OKI-179 (OnKure). The majority are reported to be HDAC6 selective inhibitors, while OKI-179 is a synthetic analogue of largazole (5).

HDAC inhibitors as cytotoxic agents

After the DNMTs, HDACs were the next epigenetic target to receive scrutiny for drug discovery and this rapidly accelerated with the early discovery of compounds that displayed potent antiproliferative activity in human cancer cell lines that was

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3 recapitulated in tumor xenograft animal models. At the same time, the simplicity and
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6 flexibility of the HDAC pharmacophore provided medicinal chemists with the freedom to
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10 operate and design and optimize independent lead series. Since the first-in-human
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13 experiments with vorinostat in 2000, HDAC inhibitors have been investigated in nearly a
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17 thousand clinical trials, primarily in oncology but numerous other indications as well. It is
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21 fair to say the accumulated clinical experience from the last two decades is
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23
24
25 disappointing as it did not fulfill the promise of the earlier data from in vitro and in vivo
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27
28 models. Efficacy with an acceptable margin of safety and tolerability has mainly been
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30
31 observed in certain hematological cancers.^{75,76} To date, we have four approvals for T-
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34 cell lymphoma, one for multiple myeloma and one for combination therapy against
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37 breast cancer, and the HDAC inhibitors are not first-line therapy against any of these
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41 indications.
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46 What HDAC inhibitors actually do in cells is an extremely complicated question.
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50 Microarray experiments indicate that <10% of the genome undergoes significantly
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53 altered expression upon treatment with a HDAC inhibitor.⁷⁷ In a cancer cell, these
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3 perturbations appear to disrupt their metastable state and drive the cell towards non-
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7 proliferative fates involving a combination of differentiation, immunomodulation,
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10 chromatin instability, reduced DNA damage repair, reactive oxygen species production,
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14 cell cycle arrest, apoptosis, autophagy, and the reduction of angiogenesis and cell
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17 migration.⁷⁸ These effects are most strongly felt in hematological cancers, perhaps due
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20 to the fact that the tumor is derived in a complex process of hematopoiesis from stem
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24 cells. Epigenetic programming plays a heavy role in this transformation, and the blood
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28 cell lineages appear to be more plastic and susceptible to reprogramming compared to
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31 more terminally differentiated cancer types. The particular success in the treatment of T-
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34 cell lymphoma is strongly linked to immunomodulation, as HDAC inhibitors cause a
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38 reduction in the 'cytokine storm' characteristic of this disorder.⁷⁹ Meanwhile, it has
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42 proven challenging to find a therapeutic window that allows the higher dosing needed
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46 against more aggressive cancers without compromising patient tolerability.⁸⁰ Resistance
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49 to HDAC inhibitors is a further issue,⁸¹ mainly arising via compensating changes in
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52 HAT/HDAC expression levels, the induction of p21 (CIP1/WAF1) and thioredoxin, and
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56 drug efflux by ABC transporters.
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4 The first clinical candidates vorinostat and romidepsin have limited oral bioavailability
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6
7 and their trials required intravenous administration. Romidepsin, with its complex
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9
10 structure, was considered intractable for analogue generation although this would
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12
13 eventually change through the design of efficient total synthesis routes by academic
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15
16 groups.⁸² While vorinostat is a much simpler molecule and an oral formulation was later
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18
19 developed, it suffers from extensive metabolism through hydroxamic acid
20
21
22 glucuronidation and oxidative cleavage of the aliphatic methylene chain. For fast
23
24
25 followers, the main challenge was to improve target affinity and the pharmacokinetic
26
27
28 liabilities and both aspects were successfully addressed in second generation HDAC
29
30
31 inhibitors. However, the primary focus was cancer, in which the cytotoxic activity is
32
33
34 largely driven by the inhibition of HDAC1-3. Thus, medicinal chemistry lead optimization
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36
37 consciously or subconsciously selected for compounds with high activity against these
38
39
40 nuclear isoforms. In the clinic, both the older and the newer compounds exhibit a similar
41
42
43 pattern of dose limiting toxicities and adverse events dominated by fatigue, diarrhea,
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45
46 bone marrow toxicity, thrombocytopenia and in some cases cardiac abnormalities.
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49 Since these are observed regardless of the nature of the inhibitor, they are likely to be
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3 on-target effects due to systemic HDAC inhibition rather than compound specific
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7 idiosyncrasies. The pleiotropic nature of the cellular response to HDAC inhibition is
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10 indicative of a global disruption of gene expression, which leads to the following
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14 conclusions:

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27 *1. The clinical effects are driven by HDAC1, HDAC2 and HDAC3 inhibition*
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32 The inhibition of these isoforms increases the acetylation of histones and other nuclear
33
34
35 proteins, leading to gene activation through increased accessibility of DNA by RNA pol
36
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38
39 II and the promotion of protein-protein interactions between bromodomain containing
40
41
42 proteins and acetyllysine residues. Our current portfolio of clinical candidates (Tables 3
43
44
45 and 4) is unanimously identical in its high activity against HDAC1-3 and we believe this
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49 is the reason why compounds are not particularly differentiated from one another in their
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53 biological effects.
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4 *2. The inhibition of isoforms in addition to HDAC1-3 by current candidates is likely to be*
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6
7 *undesirable*
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10
11 The clinical compounds primarily vary in their selectivity between the nuclear isoforms
12
13 HDAC1-3 and the cytoplasmic HDAC6. Some inhibit both the nuclear and cytoplasmic
14
15 HDAC1-3 and the cytoplasmic HDAC6. Some inhibit both the nuclear and cytoplasmic
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17 enzymes strongly (e.g. vorinostat and fast followers) while others are either selective for
18
19 HDAC1-3 (e.g. romidepsin, *ortho*-aminoanilides) or HDAC6 (e.g. ricolinostat). However,
20
21 HDAC1-3 (e.g. romidepsin, *ortho*-aminoanilides) or HDAC6 (e.g. ricolinostat). However,
22
23 these differences have not translated into major shifts in therapeutic application or
24
25 dramatic clinical benefit for one compound over another. Meanwhile, a lack of activity
26
27 observed in enzyme assays against HDAC4, HDAC5, HDAC7 and HDAC9 is a
28
29 misleading measure of selectivity, as a compound may still be capable of binding to the
30
31 active sites and thereby abrogate their non-enzymatic role in protein scaffolding. As for
32
33 HDAC8, HDAC10 and HDAC11, the traditional assays indicate differing levels of
34
35 inhibition that may again be of questionable relevance given their preference for non-
36
37 acetyllysine substrates. Although there are fine distinctions in biochemical in vitro
38
39 selectivity between the clinical inhibitors in Tables 3 and 4, this may conceal a broader
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3 in vivo spectrum of activity against the majority of isoforms. While the inhibition of
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7 isoforms other than HDAC1-3 may be beneficial, in nonselective compounds any
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10 advantage that accrues is likely to be swamped out by the side effects arising from
11
12
13 HDAC1-3 inhibition. The history of medicinal chemistry suggests that improved target
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16
17 selectivity usually leads to a superior safety profile, and this is likely to hold true for
18
19
20 HDACs as well.
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25 *3. The clinical compounds are cytotoxic agents*

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30 Although HDAC inhibitors are a modern chapter in drug discovery, their phenotypic
31
32
33 effects are widespread and not restricted to a particular cellular pathway or
34
35
36 compartment. Their activity profile as drugs, at least in its present manifestation, is
37
38
39 reminiscent of classical cytotoxic agents rather than a targeted therapy. In their account
40
41
42
43 of the discovery of vorinostat (SAHA),⁴⁷ Breslow and Marks made these prescient
44
45
46
47 comments: 'Even if some increase or decrease in transcription of particular genes is
48
49
50 helpful in the anticancer effects of SAHA, as has been proven to be the case, a
51
52
53
54 continuing long-term modulation induced by a drug that is slowly released from the
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4 receptor pocket—and indeed might bind reasonably strongly to other sites—could
5
6
7 cause undesirable effects. SAHA has hit the happy medium. It is potent enough to be
8
9
10 useful and tolerated in patients. If the dosing is intermittent, such as not to maintain a
11
12
13 continuous ‘therapeutic’ level of SAHA, it can be released from the binding site
14
15
16 periodically so as to allow the deacetylation activities in cells. This is a general
17
18
19 consideration, which could well be true of many other medicinal compounds. Thus, it is
20
21
22 probably a mistake for medicinal chemists to set out first to find the most potent
23
24
25 compound they can achieve in a series and then to look at any question of toxicity, as is
26
27
28 often done. As with SAHA, in other series there will be examples in which the medium
29
30
31 potency compound has enough efficacy to be a useful drug but not so much as to cause
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34 unacceptable side effects.’
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43 **Clinical performance of HDAC inhibitors**

44 45 46 47 *Monotherapy*

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52 Aside from the approvals in hematological cancers, HDAC inhibitors are under
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55 exploration in single agent Phase 2/3 clinical trials for other oncology applications as
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1
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3 well as non-cancer indications (Table 5). Among these examples, tefinostat is designed
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6
7 to have selective toxicity in liver cancer. Hepatocytes express carboxylesterase-1 that
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9
10 hydrolyzes the tefinostat ester to a pharmacologically active carboxylic acid which is
11
12
13 retained due to its ionized nature, whereas in other cells efflux of the neutral drug limits
14
15
16 exposure. Panobinostat, aside from its approval in multiple myeloma, is under
17
18
19 investigation as an immunomodulator for graft-versus-host diseases following allogeneic
20
21
22 hematopoietic stem cell transplantation.⁸³ Outside oncology, vorinostat is in early
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24
25 studies for the treatment of epilepsy⁸⁴ and Cushing's disease.⁸⁵ Meanwhile, Italfarmaco
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27
28 is pursuing the development of givinostat for Duchenne and Becker muscular
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30
31 dystrophy,⁸⁶ while Regency is targeting ricolinostat for diabetic neuropathic pain.⁸⁷
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38 Early indications from multiple myeloma clinical trials are encouraging regarding the
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41 tolerability of ricolinostat, 10-fold selective for HDAC6 over HDAC1-3, compared to
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44 inhibitors with lower selectivity.⁸⁸
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Cpd.	Organization	Indication	Status
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romidepsin	Bristol Squibb Myers	cutaneous T-cell lymphoma peripheral T-cell lymphoma HIV, +3BNC117	FDA approved FDA approved Phase 2, #03041012
vorinostat	Merck	cutaneous T-cell lymphoma epilepsy Cushing's disease breast cancer, + pembrolizumab, tamoxifen	FDA approved Phase 2, #03894826 Phase 2, #04339751 Phase 2, #04190056
tefinostat	GlaxoSmithKline	hepatocellular carcinoma	Phase 1/2, #02759601
ricolinostat	Regenacy	diabetic neuropathic pain	Phase 2, #03176472
belinostat	Onxeo, Spectrum	peripheral T-cell lymphoma	FDA approved
panobinostat	Novartis	multiple myeloma graft-versus-host disease myelodysplastic syndrome, + azacitidine	FDA, EMA approved Phase 3, #04326764 Phase 2, #03151304
pracinostat	Helsinn, Menarini	acute myeloid leukemia, + azacitidine myelodysplastic syndrome, + azacitidine	Phase 3, #03151408 Phase 2, #03151304
givinostat	Italfarmaco	Duchenne muscular dystrophy	Phase 2/3, #03373968

		polycythemia vera, +hydroxyurea	Phase 2, #00928707
abexinostat	Xynomics	follicular lymphoma	Phase 2, #03934567
		renal carcinoma, + pazopanib	Phase 3, #03592472
entinostat	Syndax	breast cancer, + exemestane	Phase 3, #02115282 and #03538171
		renal carcinoma, + IL-2	Phase 2, #03501381
tucidinostat	Chipscreen	peripheral T-cell lymphoma	CFDA approved
		B-cell lymphoma	Phase 3, #04231448
		breast cancer, + exemestane	NMPA approved
		soft-tissue sarcoma, + toripalimab	Phase 2, #04025931
domatinostat	4SC	GI cancers, +avelumab	Phase 2, #03812796

Table 5. Drug approvals and examples of Phase 2/3 trials conducted with HDAC inhibitors. For combination trials, the other drugs involved are indicated with '+'. The clinical trial is identified by the ClinicalTrials.gov NCT number.

Combination therapy

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4 The development of resistance is a major obstacle to cancer chemotherapy, and one of
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6
7 the contributing factors is the stem cell-like tumor phenotype that fosters epigenetic
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9
10 reprogramming in response to the drug. This suggests that combination with a HDAC
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12
13 inhibitor could be valuable in sensitizing cancer cells to the treatment, and many clinical
14
15
16 trials have investigated this hypothesis.^{89,90} Combination therapy of HDAC inhibitors
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19 with cytotoxic agents has not shown significant promise, and this further supports our
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21
22 conclusion that nonselective HDAC inhibitors themselves behave as cytotoxic agents.
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28 Meanwhile, the approval of panobinostat for multiple myeloma has led to multiple trials
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31 with first-line therapies against this indication such as bortezomib and lenalidomide.⁹¹
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35 Numerous studies have focused on the potential synergy between kinase and HDAC
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38 inhibitors, and a Phase 3 trial is investigating the combination of abexinostat and
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40
41 pazopanib.⁹²
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46 Promising results were observed in the treatment of postmenopausal breast cancer
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48
49 using the combination of the aromatase inhibitor exemestane and the *ortho*-
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52 aminoanilide HDAC inhibitor entinostat.⁹³ A larger Phase 3 trial in China with a similar
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3 combination of exemestane and tucidinostat reported positive results that led to the
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7 2019 NMPA approval of this combination for the treatment of breast cancer.⁹⁴ This
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9
10 landmark event represents the first approval of a HDAC inhibitor for an indication
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14 outside hematological cancers.
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18 Since both HDACs and DNMTs are transcriptional repressors, combined epigenetic
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21 therapy against these two targets is an attractive proposition. Pracinostat is in late stage
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23
24 trials in combination with the DNMT inhibitor azacitidine for the treatment of
25
26
27 myelodysplastic syndrome and newly diagnosed acute myeloid leukemia.^{95,96} At an
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31 earlier stage, biologic antibodies, including immunotherapy agents,⁹⁷ are being explored
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34
35 in combination trials with various HDAC inhibitors. Among the non-oncologic indications,
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38 the ability of HDAC inhibitors to reactivate latent reservoirs of the HIV virus to undergo
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41 replication appears to be the most promising for combination therapy. The potency of
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43
44 romidepsin in inhibiting the nuclear HDAC1-3 isoforms suggests it is a good candidate
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48 for viral transcriptional activation, and Phase 2 trials with the neutralizing antibody
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53 3BNC117 are underway.⁹⁸
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Dual mechanism agents

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8 An interesting approach to leveraging HDAC inhibitors is to take advantage of their
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11 simple three-point binding model. Since the cap region is not involved in critical contacts
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15 with the active site, it is possible to introduce an independent second pharmacophore
16
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18 here to give dual mechanism of action agents that achieve the equivalent of
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21 combination therapy within a single entity.^{99,100} The US company Curis has pioneered
22
23
24 this strategy, and their first clinical candidate CUDC-101 is a hybrid of vorinostat and
25
26
27 erlotinib with a nanomolar activity profile against both HDACs and receptor tyrosine
28
29
30 kinases.¹⁰¹ A second Curis candidate fimepinostat is a hybrid of pyrimidinyl HDAC
31
32
33 inhibitors and the PI3K inhibitor pictilisib, with nanomolar IC₅₀ values against all four
34
35
36 PI3K isoforms in addition to its HDAC inhibition. Fimepinostat has received FDA Fast
37
38
39 Track status for the treatment of relapsed or refractory diffuse large B-cell lymphoma.¹⁰²
40
41
42 It was shown to be similarly potent as romidepsin in the reversal of HIV-1 latency,
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44
45 without a negative effect on T cell proliferation.¹⁰³ Mundipharma's tinostamustine is a
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48 more recent dual mechanism of action agent related to the DNA alkylating agent
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3 bendamustine, and combines this activity with HDAC inhibition. Serendipitously, the
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7 HDAC inhibitor domatinostat was recently found to have a second target as it potently
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9
10 inhibits tubulin polymerization.¹⁰⁴ Larger late stage trials are needed before the efficacy
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14 and tolerability of these four multitargeting drugs can be evaluated in a clinical setting.
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23 **Atypical HDAC inhibitors**

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27 Not all HDAC inhibitors fully adhere to the classical zinc binding group-linker-cap model
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31 and some outliers illustrate the possibilities for such non-conformist compounds.
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34 Intriguingly, Olsen's romidepsin analogue **32** (Figure 11) has the thiol zinc binding group
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36
37 removed.¹⁰⁵ While the compound is two orders of magnitude weaker in HDAC inhibition
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41 than romidepsin, it still possesses a respectable level of activity. Since zinc binding
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43
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45 groups generally come with associated pharmacokinetic issues, this example suggests
46
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48 that it is possible to avoid them altogether if sufficient binding by other means is
49
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51 possible. Meanwhile, in the HDAC8 bound X-ray structure of Novartis's phenylalanine
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55 derivative **33**, the two aromatic rings sit within the hydrophobic substrate channel and
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3 the internal cavity (Figure 12), meaning that this inhibitor has no cap.¹⁰⁶ The X-ray
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6
7 structure shows coordination between the zinc cation and the α -aminoamide, a rare
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9
10 motif for a zinc binding group. As the internal cavity is absent in HDAC6, **33** is inactive
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14 against this isoform and moreover shows selectively for HDAC8 over HDAC1 and
15
16
17 HDAC2. The cyclopentenyl hydroxamic acid **34** is an even more minimal 'capless'
18
19
20
21 HDAC inhibitor with some selectivity for HDAC6.¹⁰⁷
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23
24

25 Two other unusual HDAC inhibitors are **35** and **36**. The benzoylhydrazide **35** is a typical
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27
28 example of this class with reported selectivity for the nuclear isoforms HDAC1-3.¹⁰⁸
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30
31
32 Although X-ray crystallographic evidence of the binding mode is unavailable, docking
33
34
35 studies suggest zinc coordination is not involved and the compound has unusual fast-
36
37
38 on/slow-off kinetics. On the other hand, the vorinostat analogue **36** highlights that the
39
40
41 linker is not just a space filling moiety. By switching from the suberoyl linker to its
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43
44 perfluorinated variant, the activity of the approved drug is virtually lost against human
45
46
47 HDACs. However, **36** gains in potency against two bacterial HDAC-like enzymes
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53 PA3774 and PA0321 from the pathogen *Pseudomonas aeruginosa*.¹⁰⁹
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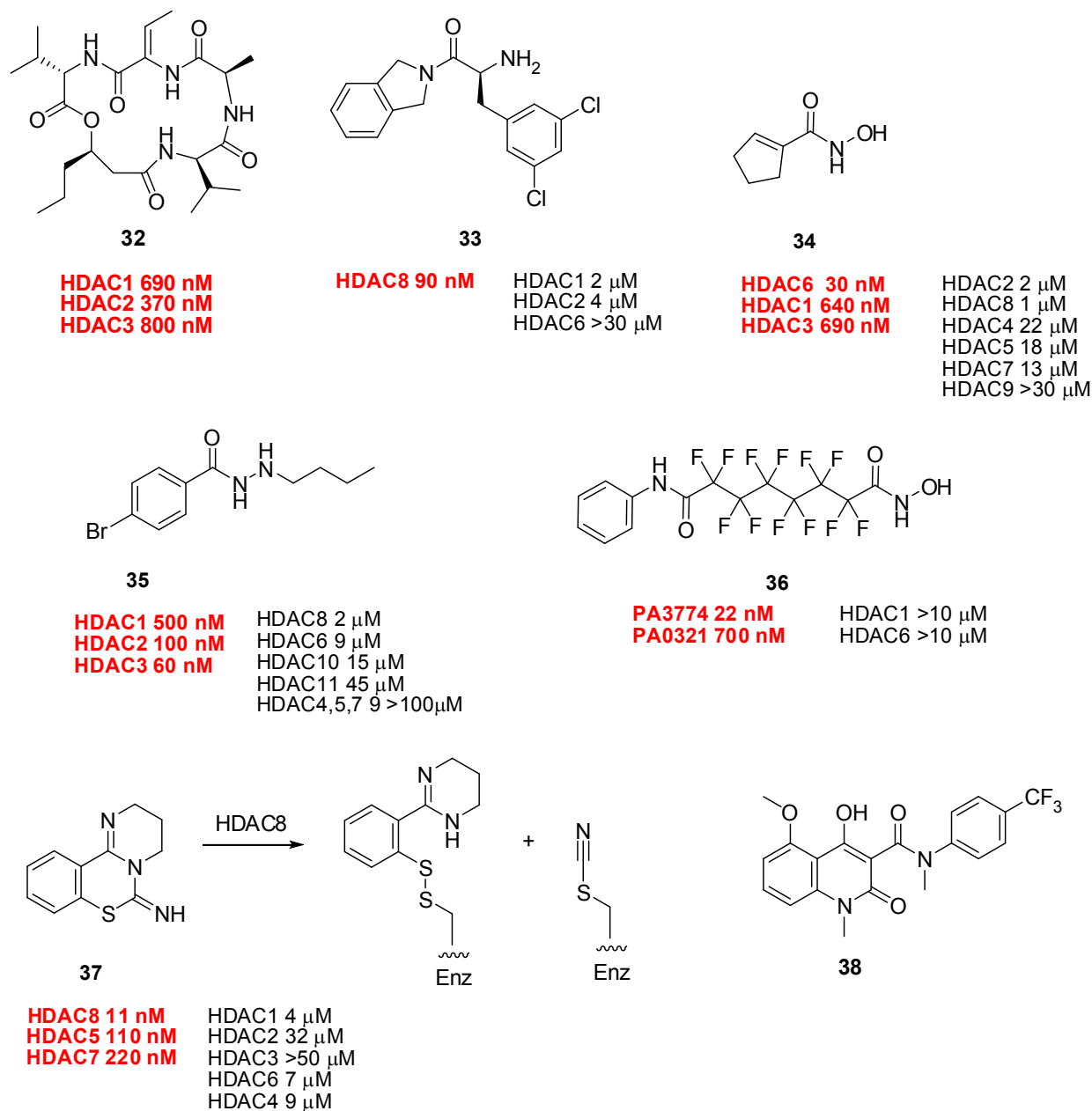


Figure 11. Some examples of unusual HDAC inhibitors. In this and subsequent figures, IC_{50} values (or in some instances K_i) against individual isoforms are given, with values below 1 μ M highlighted in red.

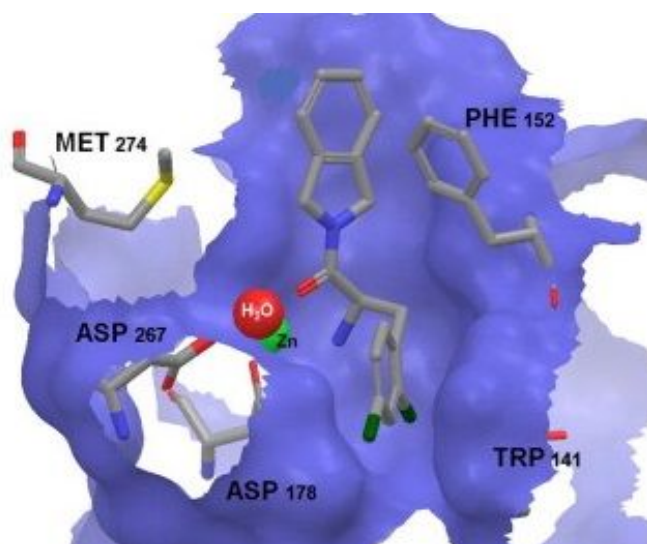


Figure 12. X-ray structure of the **33**-HDAC8 complex showing active site interactions. Reprinted with permission from ref. 106.

The heterocycle **37**, despite its compact nature and apparent lack of a linker or zinc binding group, was reported to selectively inhibit HDAC8. Further investigation revealed that **37** is an electrophilic thiol-reactive species that leads to covalent modification of the Cys28, Cys153, Cys244, Cys314 and Cys352 residues within HDAC8.¹¹⁰ Both mixed disulfide and thiocyanate adducts were identified, and enzyme inhibition was predicted to be predominantly due to reaction with the Cys153 residue within the substrate binding channel of this isoform. Perhaps the romidepsin analogue **32** has a similar mechanism of action involving allosteric modification of Cys residues by conjugate

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3 addition to the dehydroalanine residue, as this would explain why a similar largazole
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7 analogue lacking the unsaturated alkene was devoid of activity.
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11 Naturally, allosteric modulators need not adhere to the standard model for HDAC active
12
13 site binding. However, there are few examples of small molecules reported to bind with
14
15 high affinity to HDACs outside the active site. Tasquinimod (**38**) is an antagonist of the
16
17 calcium-binding protein S100A9 in Phase 3 clinical trials for castration-resistant prostate
18
19 cancer. It was also shown to bind HDAC4 with a K_d of 10-30 nM, and homology
20
21 modeling suggests allosteric binding that locks HDAC4 in an inactive conformation that
22
23 is unable to interact with HDAC3.¹¹¹ In endothelial cells, the administration of
24
25 tasquinimod induced the same phenotype of reduced vascular inflammation as HDAC4
26
27 silencing, suggesting that the interaction between HDAC4 and tasquinimod does have a
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29 functional consequence.¹¹²
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51 **Isoform selectivity**

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4 At the preclinical stage, the most exciting development in HDAC inhibitor discovery is
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6
7 the identification of compounds with an enhanced isoform selectivity profile compared to
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10 those in clinical development. At the same time, it is worth asking what selectivity
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12
13 actually means. The enzymatic activity of HDAC1, HDAC2, HDAC3 and HDAC6 can be
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16 reliably tracked with acetyllysine containing peptides and provides meaningful data on
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19 inhibitor selectivity between these isoforms, keeping in mind that in vivo kinetics while
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22 bound to other proteins and acting upon protein substrates will be different. For all other
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25 HDAC isoforms, in vitro selectivity as measured by commercial assays is of varying
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28 physiological relevance and needs to be backed up by independent demonstration of
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31 cellular target engagement through orthogonal detection methods. Despite these
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34 caveats, highly selective compounds are now available that are valuable tools for
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37 understanding the optimum specificity for cancer and neurodegenerative disorders, two
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39
40 of the most important targets for HDAC inhibitors.^{113,114} In addition, selective inhibitors
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43 are likely to be crucial for extending HDAC inhibition therapy to chronic diseases that
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46 require wider safety profiles such as inflammation,¹¹⁵ T-cell regulation,¹¹⁶ obesity,¹¹⁷
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49 fibrosis,¹¹⁸ and alcohol use disorder.¹¹⁹
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4 There are several patterns of isoform selectivity that appear particularly promising.
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7 Firstly, targeting transcriptional regulation would benefit from the selective inhibition of
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9
10 the nuclear enzymes HDAC1, HDAC2 and HDAC3 (or a subset of these three) with
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13 minimal activity against other isoforms. Conversely, selective cytoplasmic HDAC6
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16 inhibition will have its own therapeutic applications if unaccompanied by interference
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19 with nuclear isoforms. Next, there is the opportunity to focus on the turnover of non-
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21
22 acetyllysine PTMs or polyamine acetylation through the selective inhibition of HDAC8,
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25 HDAC11 or HDAC10. Finally, the active site occupancy of HDAC4, HDAC5, HDAC7 or
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28 HDAC9 (without necessarily affecting catalysis) might be sufficient for pharmacological
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31 activity and eliminate their ability to bind acetyllysine containing proteins. Moreover,
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34 more complex composite patterns of selectivity can be envisioned that combine one or
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37 more of the above options without reaching the unwanted inhibition of all eleven
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40 enzymes to a substantial degree.
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50 *HDAC1 and HDAC2*

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3 Selective inhibitors of these nuclear isoforms would be particularly useful against
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7 indications such as cancer and viral replication in which the deacetylation of histone and
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10 transcriptional regulators plays a vital role. Although the clinical compounds adequately
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13 inhibit HDAC1 and HDAC2, they are also active against some or all of the other
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16 isoforms at physiologically relevant concentrations (Tables 3 and 4) and a cleaner
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19 profile could be advantageous. Merck discovered that the addition of a 5-phenyl or 5-
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21
22 thienyl substituent to *ortho*-aminoanilide HDAC inhibitors, as exemplified by **39** and **40**
23
24
25 (Figure 13),^{120,121} or by Acetylon's ACY-957 (**41**), conferred high HDAC1/HDAC2
26
27
28 selectivity. The aryl group of the biphenyl moiety occupies the internal cavity, and in
29
30
31 these two isoforms there is a Ser107 residue while HDAC3 contains a bulkier Tyr
32
33
34 residue that limits access. Compound **39** outperformed nonselective HDAC inhibitors in
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36
37 *vitro* and *in vivo* against B-cell acute lymphoblastic leukemia, but not other B-cell-
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39
40 derived malignancies, suggesting the selective inhibition of HDAC1/HDAC2 is sufficient
41
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43 for this condition.¹²² As a single agent and in combination with the DNMT inhibitor
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45
46 azacitidine, **41** had promising results against acute myeloid leukemia, including activity
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49 against primary patient derived cells and *in vivo* xenograft models.¹²³ The PROTAC
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3 approach of recruiting an E3 ubiquitin ligase to foster degradation of a protein of interest
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6
7 has recently been applied to epigenetic targets.¹²⁴ The *ortho*-aminoanilide **42**
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10 incorporates a ligand for the von Hippel-Lindau E3 ligase.¹²⁵ Treatment of HCT116 cells
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14 with 10 μ M of **42** for 24 hours led to nearly complete degradation of HDAC1 and
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16
17 HDAC2.

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21 HDAC2 is implicated in the negative regulation of memory formation and synaptic
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HDAC2 is implicated in the negative regulation of memory formation and synaptic
plasticity in the brain, with long-term potentiation impaired in HDAC2 overexpressing
mice.¹²⁶ Treatment with vorinostat ameliorated contextual fear conditioning but had no
effect in HDAC2-deficient mice, suggesting non-redundancy between HDAC2 and
HDAC1. While the high sequence homology between these two isoforms is an obstacle
to the development of inhibitors that discriminate between the two, there are examples
of *ortho*-aminoanilides that achieve a level of kinetic selectivity. Although the Broad
Institute's BRD4884 (**43**) inhibits HDAC1 more strongly, it has a residence time half-life
of 20 minutes with HDAC1 compared to 143 minutes with HDAC2.¹²⁷ In the BRD4884-

HDAC2 X-ray structure (Figure 14), there is a bridging water molecule between the pyran oxygen and the enzyme that provides additional stabilization to the complex.

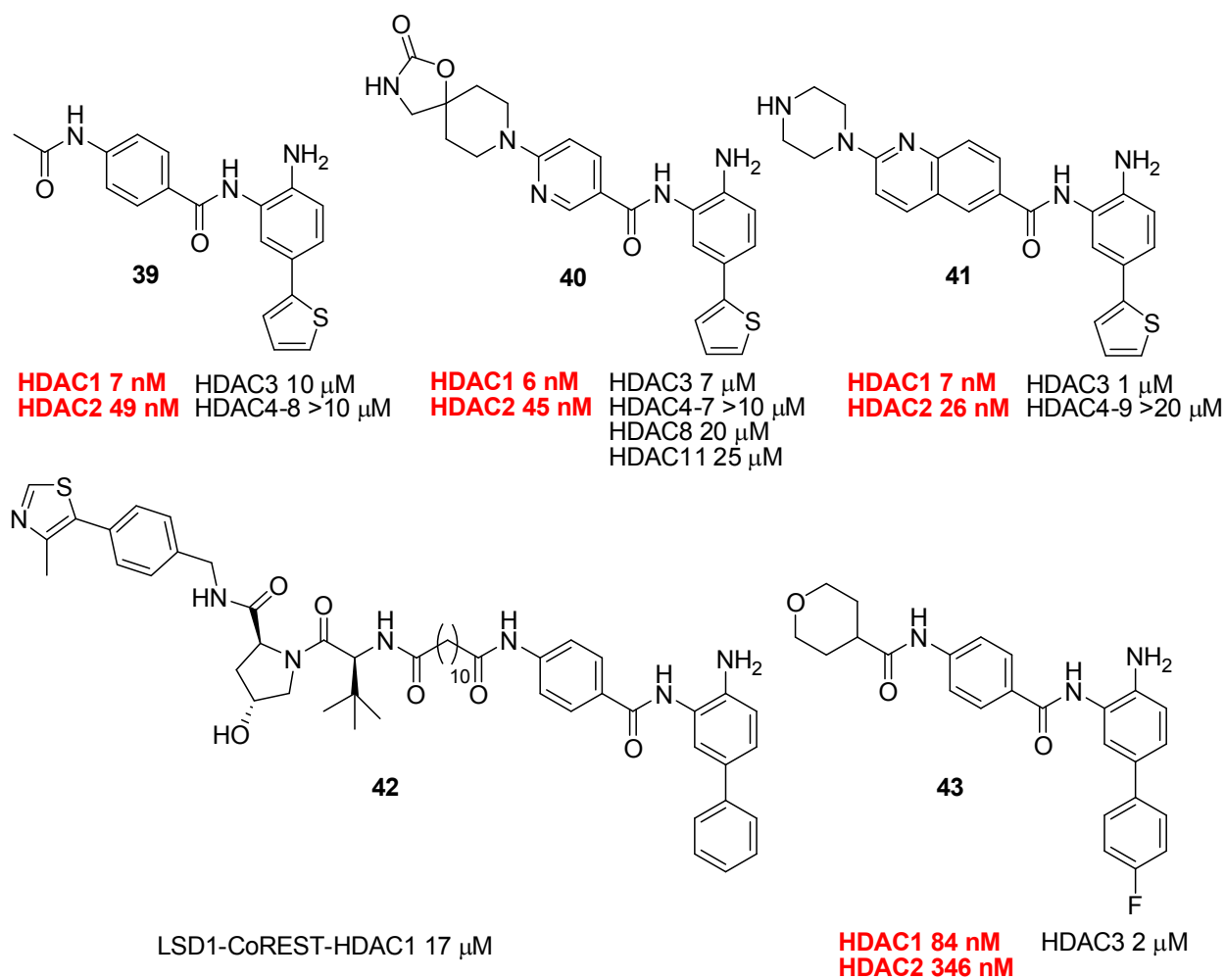


Figure 13. Examples of biaryl *ortho*-aminoanilide selective HDAC1/HDAC2 inhibitors.

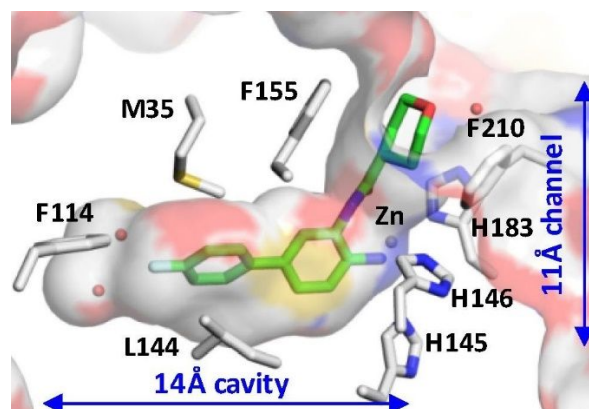


Figure 14. The X-ray structure of **43**-HDAC2 is typical of *ortho*-aminoanilides. Bidentate coordination is observed with the zinc cation, while the addition of a 5-aryl substituent enables occupancy of the lateral internal 14 Å cavity. Reprinted with permission from ref. 127.

HDAC3

HDAC3 exists in multiprotein complexes that contain NCoR/SMRT which are distinct from the repressor complexes occupied by HDAC1/HDAC2. Furthermore, the selective inhibition of HDAC3 offers the ability to indirectly modulate the tissue-specific HDAC4/HDAC5/HDAC7/HDAC9, since they recruit acetyllysine containing substrates for hydrolysis by HDAC3. The design of selective HDAC3 inhibitors has recently been

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3 comprehensively reviewed.¹²⁸ The 4-fluoro derivative (**44**, Figure 15) of the clinical
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6 candidate tacedinaline has a markedly HDAC3 selective profile compared to the latter
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9 attributed to both steric and electronic effects.¹²⁹ The more extended cap in RGFP996
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13 (**45**) from Repligen results in even higher selectivity for HDAC3 over other isoforms.
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16 More recently, the amide bearing HDAC3 selective inhibitor **46** has been reported,¹³⁰
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19 which will be useful in teasing out any compound class specific properties of the *ortho*-
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24 aminoanilides **44** and **45**.
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29 The availability of HDAC3 selective probes has proven to be illuminating in a number of
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32 in vivo disease models. In CNS applications, **45** has promoted the extinction of drug-
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35 seeking behavior,¹³¹ induced memory enhancement,¹³² reduced cognitive decline in
36
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38 Huntington's and Alzheimer's disease,^{133,134} and was protective in a stroke model.¹³⁵
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42 Furthermore, **45** activated brown and beige fat suggesting potential cardiovascular
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45 applications arising through the involvement of HDAC3 with the transcriptional
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48 coactivator PRDM16.¹³⁶ Meanwhile, **44** was shown to reactivate HIV-1 replication from
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53 resting CD4+ T cells isolated from antiretroviral-treated, aviremic HIV+ patients,¹³⁷ and
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to suppress pancreatic islet inflammation and β -cell apoptosis in non-obese diabetic mice.¹³⁸

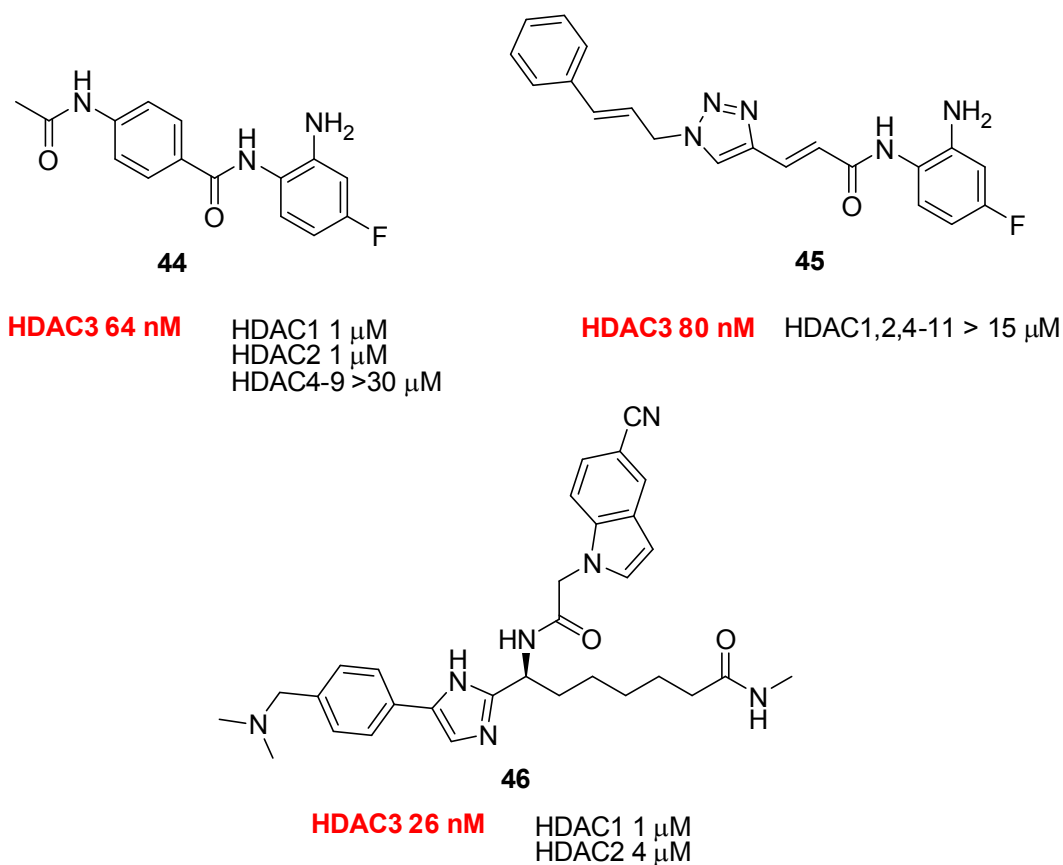


Figure 15. Examples of selective HDAC3 inhibitors.

HDAC6

The inhibition of HDAC1-3 is critical for achieving a truly epigenetic effect involving transcriptional regulation in the nucleus. On the other hand, orthogonal inhibition of the

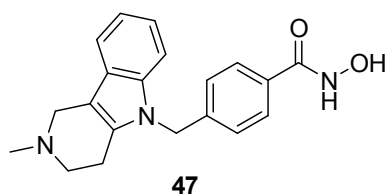
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3 major cytoplasmic isoform HDAC6 is potentially valuable against a host of human
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7 diseases. HDAC6 knockout mice are viable and fertile, and although abnormalities were
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10 reported in dopaminergic signaling,¹³⁹ this is in stark contrast with the lethality or clearly
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13 defective phenotypes observed with other isoforms. This suggests that a highly
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17 selective HDAC6 inhibitor would be well tolerated and avoids the on-target side effects
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20 of more promiscuous compounds. The substrate channel in HDAC6's second catalytic
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23 domain (CD2) is wider and shallower compared to HDAC1-3, favoring compounds with
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26 shorter linkers and larger, extended caps. However, because of similarities in the active
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29 sites between HDAC6 and HDAC8, discriminating between these two isoforms is more
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33 challenging. Kozikowski has pioneered the development of HDAC6-selective
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major cytoplasmic isoform HDAC6 is potentially valuable against a host of human diseases. HDAC6 knockout mice are viable and fertile, and although abnormalities were reported in dopaminergic signaling,¹³⁹ this is in stark contrast with the lethality or clearly defective phenotypes observed with other isoforms. This suggests that a highly selective HDAC6 inhibitor would be well tolerated and avoids the on-target side effects of more promiscuous compounds. The substrate channel in HDAC6's second catalytic domain (CD2) is wider and shallower compared to HDAC1-3, favoring compounds with shorter linkers and larger, extended caps. However, because of similarities in the active sites between HDAC6 and HDAC8, discriminating between these two isoforms is more challenging. Kozikowski has pioneered the development of HDAC6-selective arylhydroxamic acid inhibitors such as tubastatin A (**47**, Figure 16) which is widely employed as a tool compound.¹⁴⁰ Kozikowski's SW-100 (**48**) and Liou's MPT0G211 (**49**) are examples of more recent brain-penetrant inhibitors with a superior HDAC6/HDAC8 selectivity profile compared to tubastatin A.^{141,142} Instead of a phenyl linker, Acetylon's ACY-1083 (**50**) contains a pyrimidinyl ring and is reported to be > 250-fold selective for HDAC6 over other isoforms.¹⁴³

A PROTAC example with a nonselective hydroxamic acid HDAC inhibitor linked to a cereblon ligand nevertheless led to the selective degradation of HDAC6.¹⁴⁴

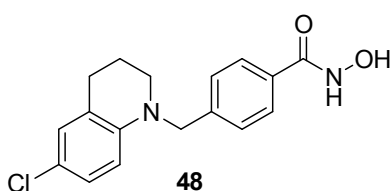
Subsequently, the group reported **51** in which the HDAC6 selective scaffold of nexturastat A was affixed to the cereblon ligand pomalidomide.¹⁴⁵ In MM1S cells, **51**

had a DC₅₀ of 1.6 nM for the selective degradation of HDAC6.



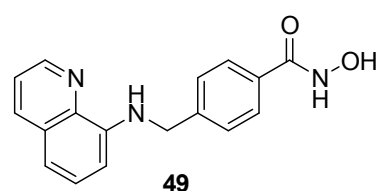
HDAC6 4 nM
HDAC8 755 nM
HDAC7 306 nM
HDAC9 739 nM

HDAC1	8 μM
HDAC2	19 μM
HDAC3	8 μM
HDAC4	3 μM
HDAC5	1 μM
HDAC10	25 μM
HDAC11	16 μM



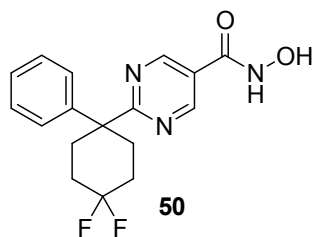
HDAC6 2 nM

HDAC1	5 μM
HDAC2	33 μM
HDAC3	30 μM
HDAC8	4 μM
HDAC4	11 μM
HDAC5	4 μM
HDAC7	5 μM
HDAC9	4 μM
HDAC10	26 μM
HDAC11	6 μM

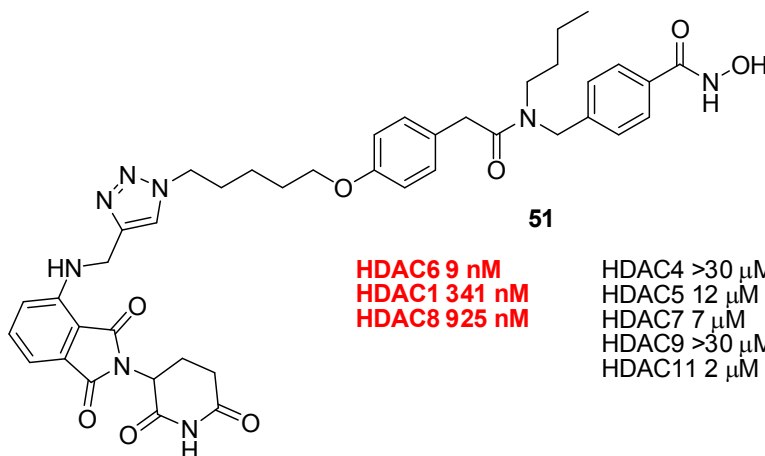


HDAC6 0.3 nM
HDAC7 709 nM
HDAC11 376 nM

HDAC1	10 μM
HDAC2	13 μM
HDAC3	8 μM
HDAC8	1 μM
HDAC4	4 μM
HDAC5	3 μM
HDAC9	2 μM
HDAC10	10 μM



HDAC6 3 nM



HDAC6 9 nM
HDAC1 341 nM
HDAC8 925 nM

HDAC4 >30 μM
 HDAC5 12 μM
 HDAC7 7 μM
 HDAC9 >30 μM
 HDAC11 2 μM

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3 **Figure 16.** Examples of selective HDAC6 inhibitors. See also the HDAC10 discussion
4 on tubastatin (**47**) and analogues.
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11 X-ray crystallographic studies with HDAC6 selective arylhydroxamic acids indicate
12 mono-coordination within the shallower active site compared to the more common
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14 bidentate zinc coordination observed against other isoforms.¹⁴⁶ Meanwhile, the aryl
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16 group engages in π - π interactions with the substrate channel while the cap extends into
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18 the solvent exposed enzyme rim, as illustrated by the binding of **50** to HDAC6 (Figure
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29 17). Recent studies (see below), suggest that tubastatin and related compounds with a
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31 basic amine in the linker or cap region are also capable of inhibiting the
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33 acetylspermidine hydrolysis activity of HDAC10.
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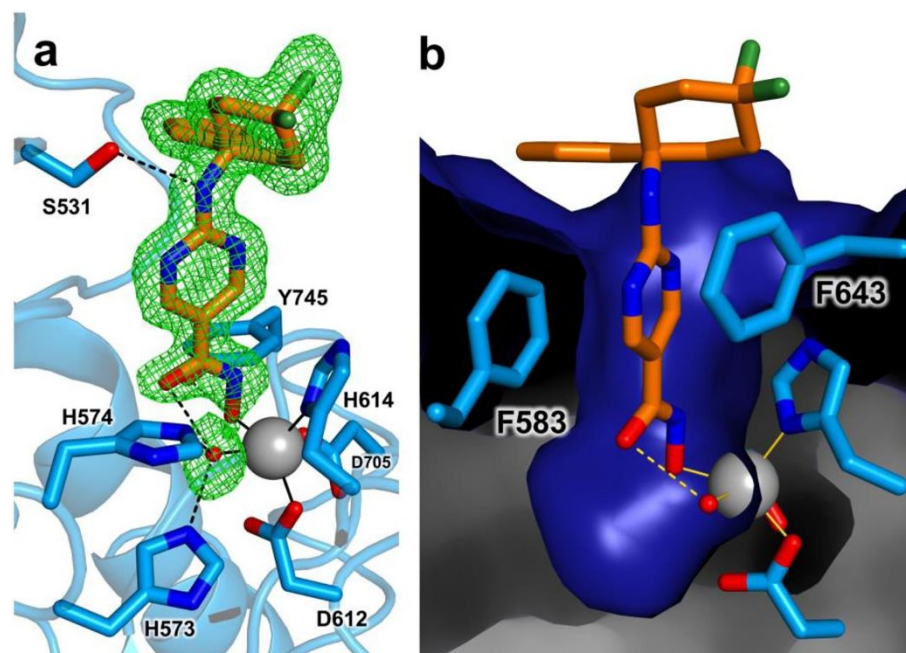


Figure 17. a) Simulated annealing omit map of the 50-HDAC6 (catalytic domain 2) binding. The hydroxamic acid engages in mono-coordinate interactions with the zinc cation while the carbonyl group hydrogen bonds the active site water involved in amide hydrolysis. The linker makes an additional hydrogen bond with Ser531. b) Cut-away view of the active site surface highlighting the zinc coordination polyhedron and the aromatic interactions between the inhibitor and the substrate binding channel. Reprinted with permission from ref. 146.

The inhibition of HDAC6 has been extensively studied in cell-based and in vivo disease models, although some examples involved compounds with lower selectivity profiles than 47-51 and phenotypic effects may be due to residual effects against other HDAC isoforms. Robust data has been obtained in animal models for cancer and

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3 neurodegenerative disorders, with early stage clinical trials ongoing for the treatment of
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7 hematological malignancies, solid tumors and Huntington's disease, as well as
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10 Regency's planned Phase 2 trial with ricolinostat for diabetic neuropathic pain.^{147,148} In
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14 addition, earlier preclinical studies are promising against a number of other
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17 indications.¹⁴⁹
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26 *HDAC8*

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31 The challenge with HDAC8 inhibition is to avoid hitting the other nuclear isoforms
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34 HDAC1-3 as well as the cytoplasmic HDAC6.¹⁵⁰ With regards to the latter, *ortho*- or
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37 *meta*-substituted aromatic linkers are helpful, compared to the *para*-substitution typical
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41 in HDAC6 selective inhibitors. Pharmacyclic's PCI-34051 (**52**, Figure 18) was the first to
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45 exhibit high selectivity for HDAC8 and is the most widely used tool compound for this
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48 isoform.¹⁵¹ Newer examples include Huang's cinnamoyl terphenylhydroxamic acid WK2-
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52 16 (**53**) and Beeler's phenylalanine derived hydroxamic acid **54**.^{152,153} In animal models,
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54
55 selective HDAC8 inhibitors have shown promise in neuroblastoma¹⁵⁴ as well as different
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inflammatory settings such as hypertensive inflammation,¹⁵⁵ neuroinflammation¹⁵⁶ and airway inflammation.¹⁵⁷ Recently, **52** was shown to resensitize melanoma tumor xenograft cells to inhibition by the tyrosine kinase inhibitor erlotinib.¹⁵⁸

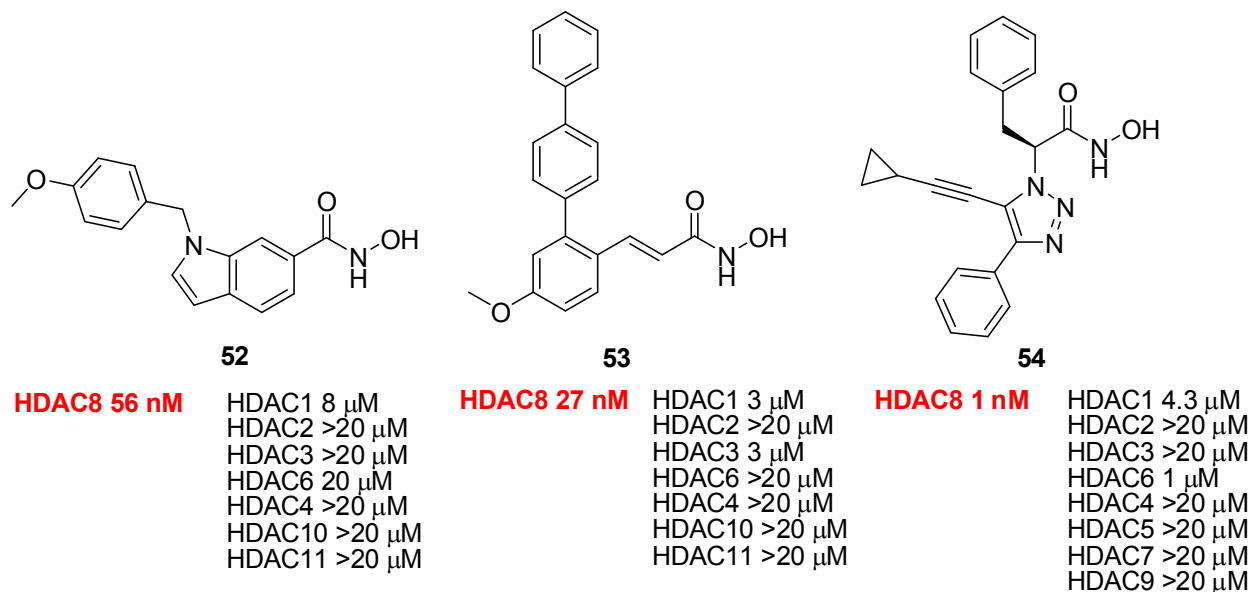
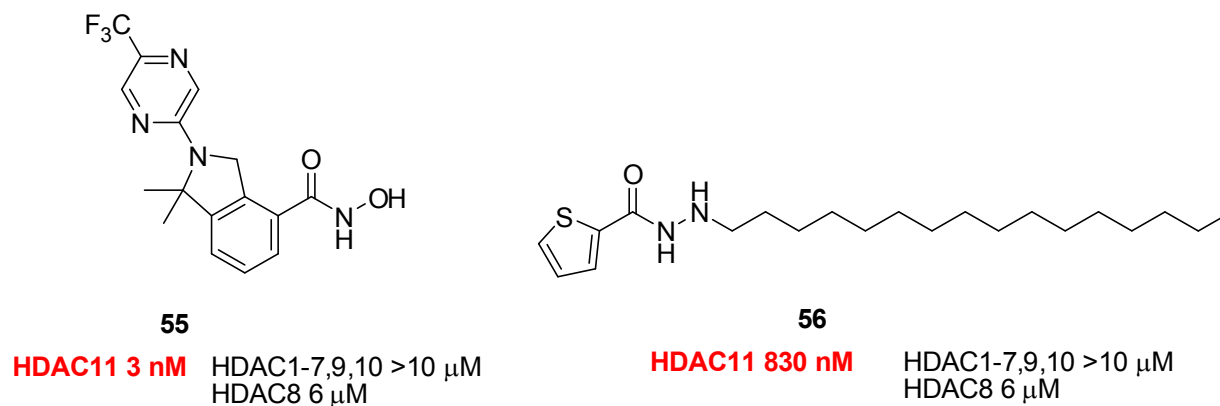


Figure 18. Examples of selective HDAC8 inhibitors.

HDAC11

The first selective HDAC11 inhibitor FT895 (**55**, Figure 19) was recently reported by FORMA, and modeling suggests that the pyrazine ring extends into the internal cavity adjacent to the zinc cation.¹⁵⁹ While fluorescent acetyllysine peptide substrates were

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3 used in FORMA's enzymatic assays, Lin employed a potentially more relevant
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7 myristoyllysine peptide with HPLC monitoring to discover acylhydrazide inhibitor **56**.¹⁶⁰
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10 In this assay, **55** and **56** were of similar potency in inhibiting demyristoylation.
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13 Presumably, **56** binds to HDAC11 with the alkyl chain occupying the internal cavity.
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17 With the availability of such probes, in vivo investigations will become possible to
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21 validate the importance of HDAC11 as a therapeutic target.
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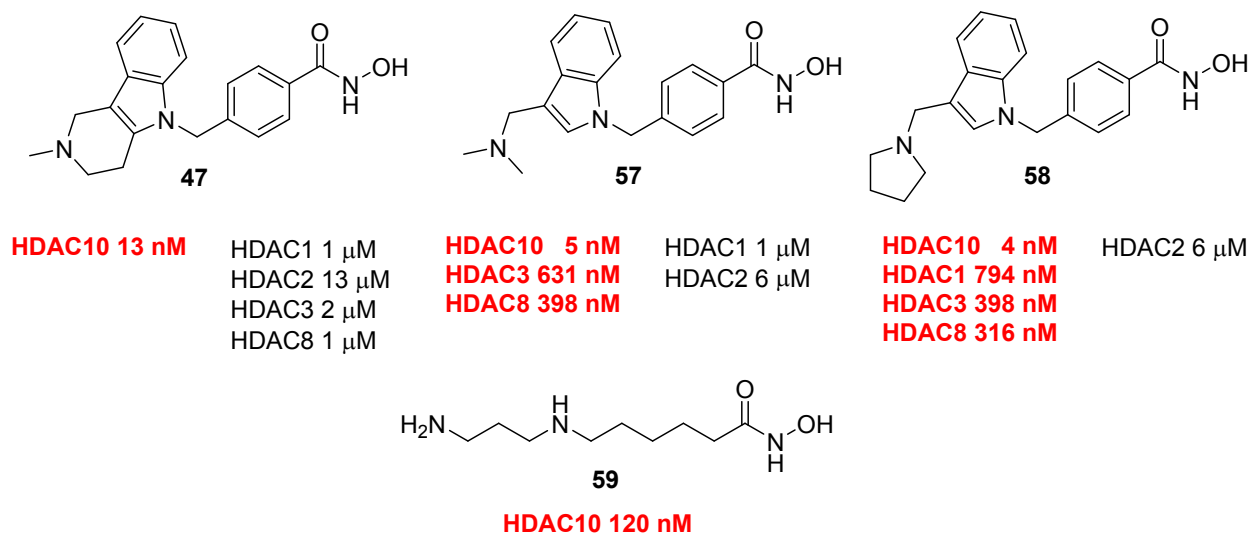


42
43 **Figure 19.** Examples of selective HDAC11 inhibitors.
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46 47 *HDAC10*

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51 The profiling of approved and clinical candidate HDAC inhibitors indicates that many
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55 strongly inhibit HDAC10 in assay kits employing trifluoroacetyllysine substrates.
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4 Although we now know that HDAC10 is a small molecule polyamine deacetylase, the
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7 assay results indicate that many HDAC inhibitors are capable of entering and occupying
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10 the substrate channel. Recently, instead of the conventional monitoring of product
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13 formation, Miller employed a FRET ligand displacement assay. In this assay, the
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16 HDAC6 selective inhibitor tubastatin A (**47**) actually bound more strongly to HDAC10
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19 than HDAC6.¹⁶¹ Since tubastatin A was much more effective than other HDAC inhibitors
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22 tested, it is likely that the molecule's amine forms a favorable electrostatic interaction
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25 with the glutamate gatekeeper residue unique to HDAC10. From a library of tubastatin A
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28 analogues, compounds **57** and **58** (Figure 20) were slightly more potent in HDAC10
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31 binding, perhaps due to a greater rotational flexibility to accommodate interaction with
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34 the glutamate. Further improvements will be needed to ensure HDAC6/HDAC10
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37 selectivity with these tubastatin A based compounds.
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23 **Figure 20.** Tubastatin A (**47**), analogues **57** and **58**, and the spermidine hydroxamic acid
 24 **59**. Note that IC_{50} values for **47**, **57** and **58** correspond to a FRET displacement assay
 25 for HDAC10 and enzymatic assays for other isoforms. The value for **59** comes from an
 26 assay monitoring *N*-acetylputrescine hydrolysis.
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Christianson examined a number of spermidine derivatives as potential inhibitors, using
 an enzymatic assay based on *N*-acetylputrescine hydrolysis. The hydroxamic acid **59** is
 a submicromolar HDAC10 inhibitor, and the X-ray structure (Figure 21) of **59** complexed
 to *Danio rerio* (zebrafish) HDAC10 has provided useful information on the binding
 interactions. The hydroxamic acid engages in bidentate coordination with the zinc
 cation, while the methylene chain features a bifurcated hydrogen bond between the
 secondary amine NH proton and the gatekeeper glutamate residue.¹⁶²

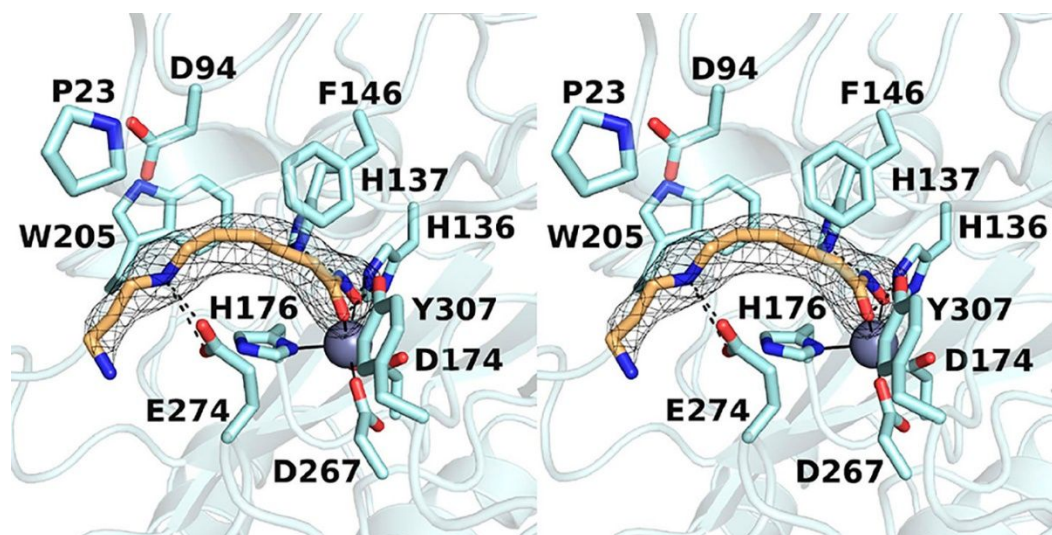


Figure 21. Polder omit map of **59** bound to the active site of HDAC10. The hydroxamic acid is coordinated as a bidentate ligand to the zinc cation, while the NH proton engages in hydrogen bonding with the Glu274 gatekeeper residue. Reprinted from ref. 162.

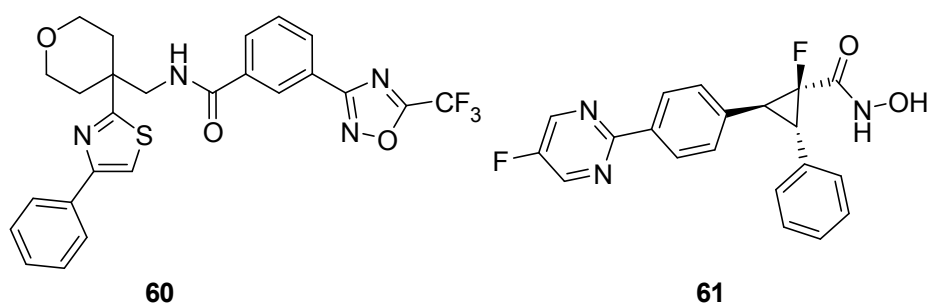
HDAC4/HDAC5/HDAC7/HDAC9

Since these isoforms have low catalytic activity, assays have employed mutant enzymes or reactive trifluoroacetyllysine substrates. While the physiological relevance of such data is questionable, it does give an idea of active site occupancy. The highly selective inhibitor TMP269 (**60**, Figure 21) was reported by Tempero and contains an

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3 unusual oxadiazole zinc binding group.¹⁶³ The X-ray structure of **60** bound to HDAC7
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6 revealed a U-shaped conformation with interactions between a fluorine and the
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10 oxadiazole oxygen with the zinc, at relatively long distances of 2.7 Å and 3.0 Å
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14 respectively compared to the ~2 Å observed with hydroxamic acids. Thus, the binding
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17 involves weak electrostatic interactions with zinc rather than direct coordination.
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21 Because these HDACs have an active site tyrosine (Figure 2) replaced by the smaller
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24 histidine, the more roomy substrate binding channel is able to accommodate the bulky
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27 trifluoromethyloxadiazole unlike the other isoforms, leading to high selectivity. In mouse
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31 models, **60** demonstrated protective activity against pulmonary arterial hypertension and
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34 cerebral ischemia/reperfusion injury.^{164,165} The structurally related analogue TMP195
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38 reduced mycobacterial infection in a zebrafish embryo model for tuberculosis.¹⁶⁶
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43 The CHDI Foundation reported a more conventional hydroxamic acid inhibitor **61** but
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46 with an unusual chiral cyclopropane scaffold.¹⁶⁷ The first generation compound lacking
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49 the cyclopropyl fluorine substituent had poorer pharmacokinetic properties due to
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52 glucuronidation. An X-ray structure of *des*-fluoro-**61** with HDAC4 (Figure 22) indicates
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3 bidentate coordination of the hydroxamic acid with the phenyl ring in a lower pocket
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7 engaged in π -stacking interactions. More recently, CHDI have disclosed the aromatic
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10 hydroxamic acid CHDI-390576 (**62**) with good plasma, muscle and brain exposure in
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14 vivo upon oral administration in mice.¹⁶⁸
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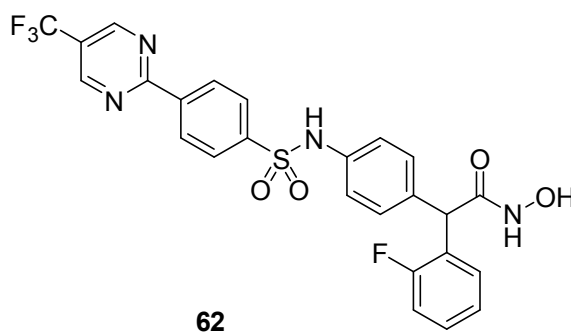
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HDAC4 126 nM
HDAC5 80 nM
HDAC7 36 nM
HDAC9 19 nM

HDAC1 >23 μ M
HDAC2 >19 μ M
HDAC3 >43 μ M
HDAC8 4 μ M
HDAC6 2 μ M
HDAC10 >10 μ M
HDAC11 >19 μ M

HDAC4 10 nM
HDAC5 10 nM
HDAC7 30 nM
HDAC9 60 nM
HDAC8 280 nM

HDAC1 14 μ M
HDAC2 >50 μ M
HDAC3 7 μ M
HDAC8 4 μ M
HDAC6 3 μ M



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HDAC4 54 nM
HDAC5 60 nM
HDAC7 31 nM
HDAC9 50 nM

HDAC1 40 μ M
HDAC2 >50 μ M
HDAC3 26 μ M
HDAC8 9 μ M
HDAC6 6 μ M

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Figure 21. Examples of selective HDAC4/HDAC5/HDAC7/HDAC9 inhibitors.

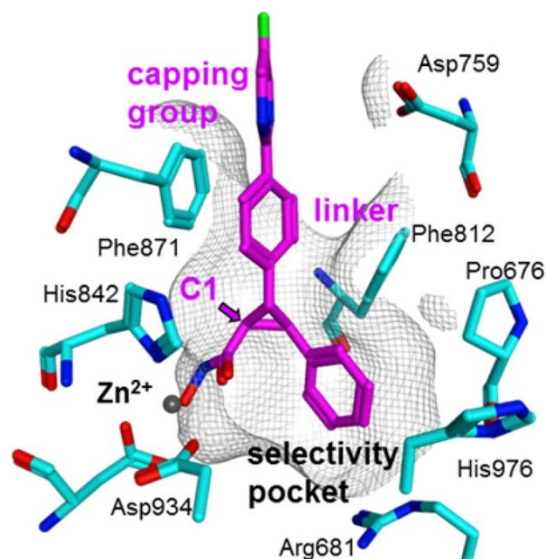


Figure 22. The binding interactions of *des*-fluoro-61 (with H instead of F at C1) within the HDAC4 active site, showing the occupancy of the selectivity pocket by the phenyl ring. Reprinted from ref. 167.

Summary

The timeline from bench to bedside was remarkably speedy for HDACs. The clinical trials of a HDAC inhibitor began in 2000, only four years after the first mammalian HDAC was characterized. In the last twenty years, over 30 HDAC inhibitors have entered clinical development with five regulatory approvals. Nevertheless, these medicinal chemistry successes have occurred despite the biological complexity of the target. Although there are only eleven human HDACs, this small number carries out a

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3 set of at least five cellular functions: nuclear lysine deacetylation (HDAC1/2/3),
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6 cytoplasmic lysine deacetylation (HDAC6), fatty acid deacylation (HDAC8/11),
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10 polyamine deacylation (HDAC10) and acetyllysine recognition (HDAC4/5/7/9). We
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14 believe that selectivity will be the key to unlock the full therapeutic value of HDAC
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17 inhibitors, and a toolkit of compounds is now available (Table 6) that will fuel future
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21 discoveries in HDAC biology and drug discovery.
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Isoform	Compound class	Examples	Selectivity challenge
HDAC1/2	5-aryl- <i>ortho</i> -aminoanilides	39-43	HDAC1 vs 2
HDAC3	4-fluoro- <i>ortho</i> -aminoanilides	44, 45	HDAC1/2
HDAC6	<i>para</i> -arylhydroxamic acids	47-51	HDAC8/10
HDAC8	<i>ortho/meta</i> -arylhydroxamic acids	52-54	HDAC6
HDAC11	<i>ortho/meta</i> -arylhydroxamic acids	55	HDAC8
HDAC10	basic hydroxamic acids	47, 57-59	HDAC6
HDAC4/5/7/9	oxadiazoles, α -branched hydroxamic acids	60-62	HDAC4 vs 5 vs 7 vs 9

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49 **Table 6.** Summary of the major isoform selective HDAC inhibitors, and the selectivity
50 issues faced within each class.
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3 While the next 30 years are too distant for accurate predictions, we anticipate that major
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7 advances will already occur within the next decade along many fronts including the
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10 following:

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15 • Target validation to identify the ideal HDAC inhibitor profile for specific
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18 therapeutic indications.
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- 23 • Unconventional approaches to HDAC drug discovery e.g. dual mechanism
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26 agents, PROTACs, covalent inhibitors, non-active site modulators.
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- 31 • Personalized medicine and patient stratification strategies, which are rare at the
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34 present time for HDAC inhibitor therapy.
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- 39 • Clinical optimization of HDAC dosing regimens, particularly the timing of
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42 administration for combination therapies.
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4 AUTHOR INFORMATION
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15 **Author Contributions**
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19 The manuscript was written through contributions of all authors. All authors have given
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21 approval to the final version of the manuscript.
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27 **ABBREVIATIONS**
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30 ABC, ATP-binding cassette; Abl, Abelson murine leukemia viral oncogene homolog;
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33 ARID, AT-rich interactive domain-containing protein; ATM, ataxia-telangiectasia
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36 mutated; BAX, BCL2 associated X protein; BRD, bromodomain; CAF, chromatin
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38 assembly factor; CFDA, China Food and Drug Administration; CIP, cyclin-dependent
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41 kinase inhibitory protein; CoREST, co-repressor for element-1-silencing transcription
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44 factor; DC₅₀, half maximal concentration of protein degradation; DNMT, DNA
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47 methyltransferase; E2F, E2 promoter binding factor; EMA, European Medicines Agency;
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51 ERR, estrogen-related receptor; FDA, Food and Drug Administration; FOXP, forkhead
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3 box P; FRET, fluorescence resonance energy transfer; GR, glucocorticoid receptor;
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7 GRP, glucose-regulated protein; HAT, histone acetyltransferase; HDAC, histone
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10 deacetylase; HIF, hypoxia-inducing factor; Hos, Histone deacetylase-A one similar; Hsp,
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13 heat shock protein; Ku, lupus Ku autoantigen protein; LSD, lysine-specific demethylase;
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17 MEF, myocyte enhancer factor; MiDAC, mitotic deacetylase complex; MIER, mesoderm
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21 induction early response; NCoR, nuclear receptor corepressor; NCT, national clinical
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24 trial; NES, nuclear export sequence; NLS, nuclear localization sequence; NMPA,
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27 National Medical Products Administration; NuRD, nucleosome remodeling and
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31 deacetylation; PCAF, P300/CBP-associated factor (KAT2B); PRDM, PR domain
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34 containing; PROTAC, proteolysis targeting chimera; PTM, post-translational
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38 modification; SMC, structural maintenance of chromosome; SMRT, silencing mediator
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41 of retinoic and thyroid receptors; Sin, SWI-independent; STAT, signal transducer and
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45 activator of transcription; WAF, wild-type activating fragment.
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54 Biographical Sketch

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4 **Terence C. Ho** obtained his first class Master's degree in Pharmacy (MPharm) from the
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6
7 University of East Anglia (UK) in 2017. During the course, he undertook a final year
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10 project under Professor Mark Searcey on peptide stapling of peptide chains to inhibit
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13 the p53-MDM2 protein-protein interaction for applications in oncology. In 2018, he
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15
16 started studying Medicine (MBBS) at the University of East Anglia and is currently in his
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21 second year.
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25 **Alex H. Y. Chan** received his first class Master's degree of Pharmacy (MPharm) from
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27
28 the University of East Anglia (UK) in 2017. He won the Andrew Thompson Prize for the
29
30
31 best Medicinal Chemistry research project on the design and synthesis of triazole-based
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34 HDAC inhibitors for *Schistosomiasis* therapy under the supervision of Professor
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37 Ganesan. Currently he is a second year doctoral student in Chemistry at the University
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40 of Cambridge (UK) under the supervision of Dr. Finian Leeper working on the design,
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43 synthesis and biological evaluation of thiamine pyrophosphate dependent enzyme
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46 inhibitors for malarial treatment. His research interests lie in discovering small
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53 molecules as selective drugs to combat infectious diseases.
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3 **Ganesan** (one name only) was born in Malaysia and obtained his undergraduate degree
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6
7 in Chemistry from the neighboring National University of Singapore. After graduate
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9
10 studies at the University of California-Berkeley and postdoctoral research at Harvard
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12
13 University, he returned to Singapore to work at the Centre for Natural Product
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15
16 Research. Subsequently, he was Principal Investigator at Singapore's Institute of
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19 Molecular and Cell Biology and Reader at the University of Southampton's Department
20
21
22 of Chemistry before taking up his present position. Ganesan's interests are in drug
23
24
25 discovery, natural products and epigenetics. He was a chair of the European COST
26
27
28 networks TD0905 Epigenetics: From Bench to Bedside and CM1406 Epigenetic
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31 Chemical Biology. He is a co-founder of Karus Therapeutics and sits on the IUPAC
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34 Drug Discovery and Development and the RSC Bioorganic and Medicinal Chemistry
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37 Sector committees.
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