Azetidinones as Zinc-Binding Groups to Design Selective HDAC8 Inhibitors


2-Azetidinones, commonly known as β-lactams, are well-known heterocyclic compounds. Herein we described the synthesis and biological evaluation of a series of novel β-lactams. In vitro inhibition assays against HDAC isoforms showed an interesting isoform-selectivity of these compounds towards HDAC6 and HDAC8. The isoform selectivity changed in response to modification of the azetidinone-ring nitrogen atom substituent. The presence of an N-thiomethyl group is a prerequisite for the activity of these compounds in the micromolar range towards HDAC8.

Introduction

Histone deacetylases are involved in determining the pattern of acetylation of chromatin proteins, histones, in eukaryotic cells. Since the discovery of the role of reversible acetylation of histone proteins in the regulation of gene expression,[1] 18 potential human histone deacetylases (HDACs) have been discovered. These can be divided into four classes (I–IV). Class I (HDAC1, -2, -3 and -8), class II (HDAC4, -5, -6, -7, 9, -10) and class IV (HDAC11) operate by zinc-dependent mechanisms; in contrast, class III HDACs use NAD+ as a cofactor. Classes I and IV HDACs are ubiquitously expressed, are predominantly in the nucleus, and function mainly as transcriptional corepressors that are linked to cell proliferation and survival.[2] The distribution of class II HDACs, which are able to shuttle in and out of the nucleus, is more tissue specific, suggesting distinct functions in cellular differentiation and developmental processes.[3]

Currently, a number of potential drugs are in clinical trials as HDAC inhibitors; the first FDA-approved compound was suberoylanilide hydroxamic acid (SAHA), a pan-HDACs inhibitor.[4] In terms of chemical structures, HDAC inhibitors include a wide range of scaffolds and can be classified in structural classes such as aliphatic acids, hydroxamic acids, cyclic peptides and benzamides. A well-accepted pharmacophore model for these inhibitors consists of: a) a capping group that interacts with the residues at the active site entrance; b) a zinc-binding group (ZBG) that coordinates to the catalytic metal atom; c) a linker group that binds in a hydrophobic channel and positions the ZBG and a capping group for interactions in the active site (Figure 1).

Although the connections between certain HDAC isoforms and pathophysiology are still evolving, accumulating data suggest that targeting specific HDACs might be beneficial in treating certain disease conditions, while limiting side effects.[5a–d] Hence, the emerging trends are to develop novel HDAC class-specific inhibitors. Modification of the capping group, linker and ZBG individually contribute to selectivity towards specific HDAC isoforms. In spite of significant results obtained through modification of the cap group in the development of class-selective rather than isoform-selective inhibitors, similar results through modification of the ZBG have proved more difficult to obtain.

Human HDAC8 is a class I histone deacetylase that was first cloned by members of the Pharmacyclics HDAC team in 1999 and has been identified in a variety of human cancer tissues. Earlier studies suggested that HDAC8 localized to the nucleus and was ubiquitously expressed.[6a–c] Recently, it was demonstrated that HDAC8 localized to the nucleus and was ubiquitously expressed.[6a–c] It regulates telomerase activity, and may play an important role in neuroblastoma pathogenesis.[6b] Additionally, HDAC8 is associated with the actin cytoskeleton in smooth muscle cells and regulates the contractile capacity.[8] This protein has also been linked to cancer as it is recruited by the leukemic inv[16] protein;[10] it regulates telomerase activity,[13] and siRNAs targeting HDAC8 were shown to have antitumor effects in cell culture.[12] Inhibition of the secretion of proinflammatory
cytokines such as interleukin(IL)-1beta and IL-18 from peripheral blood mononuclear cells (PBMC) has been highlighted using selective HDAC8 inhibitors like PCI-34051. [13] HDAC8 is normally inhibited by the so-called pan-inhibitors, such as valproic acid (VPA), sodium butyrate (NaBu), trapoxin, SAHA, trichostatin A (TSA), PXD-101, LBH-589, LAQ-824. [14] Furthermore, some HDAC8-selective inhibitors were discovered by serendipity or designed ad hoc using co-crystal structures of inhibitors bound to HDAC8. The first crystal structure of a mammalian HDAC was published in 2004. [12–15] Analysis of the HDAC8 crystal structure in complex with an inhibitor containing an aryl linker (CRA-A) revealed a large subpocket in the side of the hydrophobic active site channel that was not apparent in the crystal structure of HDAC8 in complex with SAHA. [15] The HDAC inhibitor PCI-34051 was rationally designed with an indole present in the linker domain to specifically target this subpocket, [13] while SB-379278A was identified by an enzyme-based high-throughput screen (Figure 2). [16]

\[ \text{SB-379278A} \]

\[ \text{PCI-34051} \]

Figure 2. Known HDAC8-selective inhibitors.

\[ \beta\text{-Lactams are a very important class of bioactive molecules. Starting from the antimicrobial potency exerted by naturally occurring bicyclic compounds (penicillins and cephalosporins), nowadays new variants with monocyclic structures (azetidinones) are displaying new and specific biological activities.} \]

[17] \[ \beta\text{-Lactam-related compounds are irreversible inhibitors of a wide range of serine proteases, including elastases, }\beta\text{-lactamases, phospholipase A2, and bacterial signal peptidases.} \]

[18] Giacomini et al. have actively contributed to the development of this field with the synthesis of monocyclic }\beta\text{-lactams as scaffolds for antibiotics against resistant bacteria,} [19] as enzymatic inhibitors against human leukocyte elastase (HLE) and matrix metallo-proteases (MMPs), [20] as antioxidants, [21] and as antiaggregating agents. [22] Some aryl- }\beta\text{-lactam derivatives have previously been evaluated for cytotoxicity against a number of human tumor and normal cell lines.} [23] In particular N-thiolated }\beta\text{-lactams were found to induce DNA damage, cell growth arrest, and apoptosis in cultured human cancer cells.} [24] Interestingly some bicyclic }\beta\text{-lactams were previously reported as HDAC inhibitors.} [25]

In a research project aimed at the identification of new selective HDAC inhibitors, we found that some azetidinone derivatives showed good affinity and specificity towards HDAC8.

The design of }\beta\text{-lactams described herein was based on the modular structure as described for SAHA (Figure 1). An azetidin-2-one ring was identified as metal-binding group, a saturated or unsaturated chain as the linker, and the capping group was typical of those seen in compounds such as SAHA or }p\text{-phenylcinnamyl esters.} [26a,b] The cap–linker module is anchored on the }\beta\text{-lactam ring at one of the two side chains; the substituent on the }\beta\text{-lactam nitrogen atom is the second source of molecular diversity and we synthesized }N\text{-H, }N\text{-OH, }N\text{-SMe and }N\text{-O-aminophenyl derivatives for a specific evaluation of the }\beta\text{-lactam core as a metal-binding group (compounds 1–9). Our findings validate }N\text{-thiomethyl-}\beta\text{-lactam as a new }Zn\text{-binding group in the design of new selective HDAC8 inhibitors.}

Results and Discussion

Chemical synthesis

The synthesis of }\beta\text{-lactams required careful design of the synthetic strategies because of the sensitivity of the four member ring to harsh reaction conditions, and regio- and chemoselectivity problems due to the presence of peculiar functional groups.}

The synthesis of azetidinone 1 was achieved as shown in Scheme 1. Starting from the commercially available 4-acetoxyazetidin-2-one, we obtained 4-benzenesulfonylazetidin-2-one.

\[ \text{Scheme 1. Reagents and conditions: a) } \text{PhSO}_2\text{Na, DMF, }0^\circ\text{C} \rightarrow \text{RT}; \text{ b) } \text{but-3-enyl-MgBr, THF, }0^\circ\text{C} \rightarrow \text{RT}; \text{ c) } \text{LiHMDSA, THF, }0^\circ\text{C} \rightarrow \text{RT}; \text{ 1. } \text{MeSO}_2\text{SMe; 2) Grubbs II (5 mol%) CH}_2\text{Cl}_2, \text{reflux.} \]

10 in 77% yield. Nucleophilic substitution with but-3-enylmagnesium bromide afforded the 4-butenyl-azetidinone 11. N-Thiorification by deprotonation with lithium hexamethyldisilazide (LiHMDSA) and subsequent reaction with MeSO$_2$SMe gave 12 in 94% yield. Cross-metathesis with N-phenylacrylamide 13 in the presence of Grubbs 2nd-generation catalyst (benzyldiene[1,3-bis(2,4,6-trimethylphenyl)-2-imidazolidinylidene]dichloro(tricyclohexylphosphine)ruthenium) afforded the racemic N-thiomethyl-azetidinone 1 in 51% yield. The two enantiomers were separated by semipreparative chiral HPLC (see Experimental Section for details).

The synthesis of azetidiones 2, 3, and 4 is shown in Scheme 2. Starting from commercially available β-alanine in the presence of an excess of LiHMDSA and allyl bromide, we obtained the 2-allyl-β-alanine 14 in 53% yield. Base-catalyzed cyclization afforded the 3-allyl-azetidinone 15 in 66% yield. Cross-metathesis with pent-4-enioic acid phenylamide 17 gave 2 in 58% yield. Compound 2 was predominantly obtained as the E isomer with only traces of the Z isomer evidenced by chiral HPLC. Again, separation of the four stereoisomers was achieved using chiral HPLC (see Experimental Section). Palladium-catalyzed hydrogenation gave the azetidinone 4 in quantitative yields. Insertion of the N-thiomethyl group in 4 is difficult because a competition occurs between the lactam and the amide group in the side chain. However, N-thiomethylation of 15 successfully gave 16, which was subsequently reacted with amide 17 to give azetidinone 3 in a 37% yield (two steps). Compound 3 was predominantly obtained as the E isomer with only trace amounts of the Z isomer, but semipreparative chiral HPLC allowed the separation of the four stereoisomers (see Experimental Section).

Cross-metathesis of 3-allyl-azetidinones 15 and 16 was successful, even with 4-vinylbiphenyl, giving compounds 7 and 8, respectively (Scheme 3).

The N-hydroxy-azetidinones 5a–b contain a hydroxamic acid scaffold in a cyclic form. Scheme 4 illustrates the preparation of N-hydroxy-azetidinone 5. Starting from methyl-4-pentenoate, enolization and subsequent hydroxymethylation with formaldehyde gave 18 in 42% yield. Acyl substitution by O-benzyl-hydroxylamine afforded derivative 19 (yield = 48%). Cyclization with diisopropylazodicarboxylate (DIAD) and triphenylphosphine resulted in azetidinone 20 in excellent yield (96%). Cross-metathesis with pent-4-enioic acid phenylamide 17 and subsequent hydrogenolysis with Pd-C gave the N-hydroxyazetidinone 5, which was separated into the two enantiomers by semipreparative chiral HPLC.

The N-o-aminophenylezidin-2-one 6 was prepared with a four-step synthesis starting from the hydroxyster 18 (Scheme 5). Cross metathesis of 18 with amide 17 resulted in compound 22 (yield = 47%). Acyl substitution with ortho-carbonylbenzoylaminoo-aniline (23) gave 24 in 67% yields. Cyclization with DIAD gave the azetidinone 25 in 77% yields. Hydrogenolysis (Pd-C) quantitatively resulted in the N-o-aminophenylazetidin-2-one 6. 1-(Biphenyl-4-ylmethylsulfanyl)azetidin-2-one 9 was obtained in 84% yield starting from the commercially available azetidin-2-one, deprotonation with LiHMDSA and reaction with 26 (Scheme 6).
The azetidinone side chain on the azetidinone ring (C3 or C4) slightly affected the activity; the side chain on the C4 position was a little more effective (cf. 1a–b vs 3a–b). Furthermore, the individual enantiomers had different inhibitory potencies against HDAC8 (cf. 3d vs 3c).

As opposed to the N-thiomethyl-azetidinones, the N-H or N-OH derivatives showed no activity against HDAC8, but some potency toward HDAC6; this is particularly apparent when 2a–b and 3a–b are compared. The presence of a C=C bond in the side chain had no influence on HDAC6 inhibition (see 2a–b vs 4). The N-o-aminophenyl group destroyed the activity against HDAC6 or HDAC8. The azetidinone 9, with no side chains but with an N-thio-p-phenyl group, confirmed the effectiveness of a sulfur atom for increased HDAC8 activity, however, isoform selectivity was lost.

**Computational analysis**

In order to explain the selectivity of the N-thiomethyl-β-lactam derivatives 1a–b, 3a–d, and 8a–b against the HDAC8 isoform, we performed a comparative structural study using three isoforms, HDAC2, HDAC7, and HDAC8, representative of class I and class II HDACs.

We speculated that the selectivity would be located within the active pocket in the channel and Zn-binding regions. The amino acids in these regions are highly conserved between class I and II HDACs, nevertheless, we turned our attention on two amino acids, Trp 141 and Met 274, that are specific to HDAC8 (Table 2). The interaction of N-thiomethyl-azetidin-2-one derivatives against class I and II HDACs were then evaluated using HDAC2, HDAC7, and HDAC8 isoforms as HDAC10 and -11, bearing unusual combinations of amino acids, are not reliable.

First, we calculated the geometry and energy of the Zn$^{2+}$ in complex with the 1-methylsulfanyl-azetidin-2-one (N-thiomethyl-azetidin-2-one) unsubstituted at the C3 and C4 positions of the ring; a bidentate coordination geometry was found where distances between the Zn$^{2+}$ ion and coordinating atoms (O and S) were 1.84 Å and 2.45 Å, respectively. This geometry was used as a starting point to superimpose the 1-methylsulfanyl-azetidin-2-one on the hydroxamic acid moiety of the inhibitors co-crystallized with the HDACs using the crystal structure of HDAC7 (PDB code: 3C0Z) and HADC8 (PDB code: 1T67) and an in-house 3D structural model of HDAC2. In the active site, the Zn$^{2+}$ coordination geometry is a square-based pyramid where the inhibitor and two aspartic acids form the base of the pyramid and a histidine is the vertex. After minimization, the 1-methylsulfanyl-azetidin-2-one loses bidentate coordination geometry in all the complexes because of unfavorable steric contacts with two conserved glycine residues (304 and 151 in HDAC8) with the methyl group and CH$_3$ on the C4 position of the azetidinone ring, respectively. We decided to use the model 1-methylsulfanyl-azetidin-2-one as a monodentate ligand, adding a water molecule as the fifth zinc coordinating atom.

Using the N-thiomethyl-azetidinone 3c as a representative ligand of the series in the configuration (R) of the C-3 position...
Selective HDAC8 Inhibitors

of the ring, we performed molecular dynamic calculations of 3c–H₂O–HDAC2, -7 and -8 complexes, to better characterize the interaction geometry. Molecular dynamics simulations were performed, and 100 structures for each complex were sampled and minimized. We calculated the mean distance between the Zn²⁺ ion and the coordinating heteroatom of the ligand, the mean number of contacts calculated between N-thiomethylazetidin-2-one ring and each nonconserved amino acid in the zinc-binding region (Leu 140, Pro 667, Trp 141), the mean number of contacts calculated between 3c and each nonconserved amino acid in the linker region (Leu 272, Leu 810, Met 274), the mean number of contacts between

Table 1. HDAC isoform selectivity profile of [lactams 1–9][a]

<table>
<thead>
<tr>
<th>Compd</th>
<th>Structure[b]</th>
<th>HDAC6 [µM]</th>
<th>HDAC8 [µM]</th>
<th>Compd</th>
<th>Structure[b]</th>
<th>HDAC6 [µM]</th>
<th>HDAC8 [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSA</td>
<td></td>
<td>0.00042</td>
<td>0.089</td>
<td>2a</td>
<td></td>
<td>132.0</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>3a</td>
<td></td>
<td>&gt;1000</td>
<td>34.3</td>
<td>2b</td>
<td></td>
<td>64.1</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>3b</td>
<td></td>
<td>&gt;1000</td>
<td>11.6</td>
<td>2c</td>
<td></td>
<td>76.0</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>3c</td>
<td></td>
<td>&gt;1000</td>
<td>33.1</td>
<td>2d</td>
<td></td>
<td>90.0</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>3d</td>
<td></td>
<td>&gt;1000</td>
<td>9.56</td>
<td>5a</td>
<td></td>
<td>132</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>1a</td>
<td></td>
<td>&gt;1000</td>
<td>10.1</td>
<td>5b</td>
<td></td>
<td>174</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>1b</td>
<td></td>
<td>&gt;1000</td>
<td>4.5</td>
<td>7a</td>
<td></td>
<td>93.3</td>
<td>&gt;1000</td>
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<tr>
<td>8a</td>
<td></td>
<td>138</td>
<td>47.1</td>
<td>7b</td>
<td></td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>8b</td>
<td></td>
<td>&gt;1000</td>
<td>30.9</td>
<td>4</td>
<td></td>
<td>74.5</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>32.2</td>
<td>24.7</td>
<td>6</td>
<td></td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

[a] See Experimental Section for assay conditions. Trichostatin A (TSA) was used as a reference compound. [b] Absolute configurations depicted are tentatively assigned on the basis of computational analysis (see text for details).

Table 2. Amino acid differences between HDAC8 and the other HDAC isoforms.

<table>
<thead>
<tr>
<th>Amino acid Class I</th>
<th>Class II</th>
<th>Class IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn-binding region</td>
<td>Trp 141</td>
<td>HDAC8</td>
</tr>
<tr>
<td></td>
<td>Leu⁶¹</td>
<td>HDAC1–3</td>
</tr>
<tr>
<td></td>
<td>Pro⁶¹</td>
<td>–</td>
</tr>
<tr>
<td>Channel region</td>
<td>Met 274</td>
<td>HDAC8</td>
</tr>
<tr>
<td></td>
<td>Leu⁶¹</td>
<td>HDAC1–3</td>
</tr>
<tr>
<td></td>
<td>Glu 272</td>
<td>–</td>
</tr>
</tbody>
</table>

[a] Numeration is variable in dependence of different HDAC isoforms.
amino acids in a range of 5 Å from the inhibitor, and $\Delta G_{\text{binding}}$ of 3c for each HDAC. Table 3 shows the molecular dynamic simulation results; superimpositions of HDAC2/HDAC8 and HDAC7/HDAC8 minima are shown in Figure 3 a and b.

Table 3. Mean distances and contacts from dynamics, and $\Delta G_{\text{binding}}$ values.

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Bond distance Zn–O/S (Å)</th>
<th>Good contacts</th>
<th>Total $\Delta G_{\text{binding}}$ [kJ mol$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDAC2</td>
<td>2.64 (O)</td>
<td>0.1</td>
<td>11.0</td>
</tr>
<tr>
<td>HDAC7</td>
<td>3.50 (S)</td>
<td>2.8</td>
<td>10.7</td>
</tr>
<tr>
<td>HDAC8</td>
<td>2.23 (O)</td>
<td>16.1</td>
<td>12.5</td>
</tr>
</tbody>
</table>

[a] Calculated from energy-minimized complexes.

The HDAC2 complex shows a more accessible space in the zinc-binding region compared with HDAC8,[28b] and as such can accommodate a larger ZBG, however, in this case the S-Me group of N-thiomethyl-azetidin-2-one is too small and does not interact with Leu140. A good number of interactions are found between 3c and Leu272 in the linker region, however, these interactions are inferior than those with Met242 in HDAC8. Moreover, azetidin-2-one makes poor contacts with Gly150 and Phe151.

Analyzing the HDAC8 complex, we noted that 3c maximizes the numbers of contacts in both regions without bad interactions and anchors the cap group to the external region of the enzyme via a H bond with Phe207 (residue not shown in the figures for clarity). Both complexes maintain a Zn$^{2+}$–carbonyl interaction similar to the natural substrate (acetylated lysine).[28a] Conversely, the S-Me group of 1-methylsulfanyl-azetidin-2-one makes bad contacts with Pro667 in HDAC7, and during the simulation 3c moves until the S atom is in the correct position to coordinate with the Zn$^{2+}$ ion. However, the S-Me group still interacts with Pro667. The total numbers of contacts between 3c and HDAC7 decreases due to the more external binding orientation in comparison with other complexes.

By measuring the $\Delta G_{\text{binding}}$ of the three minima, we can see that the order of stability of 3c–HDACs complexes is HDAC8 > HDAC7 > HDAC2, in good accordance with the isoform selectivity found in the in vitro tests. Considering the monodentate coordination geometry of this ligand with respect to a bidentate one, the lower activities of these new $\beta$-lactam ligands compared with SAHA or TSA can be justified.

Subsequently, we used the refined 3c-HDAC8 complex to dock compounds 1a, 1b, 3a, 3b, 3c, 3d, 8a, and 8b and to compare them in terms of $\Delta G_{\text{binding}}$. As shown in Figure 4a, all the inhibitors adopted the same orientation as 3c with the carbonyl coordinating to the zinc ion, thus confirming N-thiomethyl-azetidinone as a valuable zinc-binding group (ZBG).

We determined that each enantiomeric couple oriented the N-thiomethyl-azetidin-2-one ring with respect to the Zn$^{2+}$–carbonyl coordination bond: N-thiomethyl-azetidin-2-one interacts with Trp141 via the sulfur atom when the orientation of the side chain is “down”, and via the methyl group when it is “up” (Figure 4b). Compounds with “down” stereochemistry have $G_{\text{con}}$ and $\Delta G_{\text{binding}}$ values higher than the “up” stereoisomers, and we speculated that for each enantiomeric couple a “down” configuration could be assigned to the most active compound: $\Delta \Delta G_{\text{binding}}$ 1b–1a = -18 kJ mol$^{-1}$; 3b–3a = -12 kJ mol$^{-1}$; 3d–3c = -21 kJ mol$^{-1}$; 8b–8a = -38 kJ mol$^{-1}$.

Conclusions

The results reported here on the development of selective and potent HDAC inhibitors should shed light onto the connections between HDAC isoforms and pathophysiology, such as inflammation, cancer or immunological diseases, and validate the targeting of specific HDACs for the improved treatment of certain disease conditions with reduced side effects.

Monocyclic azetidin-2-ones, specifically designed using the HDAC inhibitor pharmacophore model, were shown to selectively inhibit HDAC6 and HDAC8. We were able to manipulate the isoform selectivity by modifying the substituent on the nitrogen atom of the $\beta$-lactam ring. In particular, N-thiomethyl-azetidin-2-ones showed a stringent isoform selectivity towards...
HDAC8 with activities in the micromolar range. Molecular modeling provided a structural explanation for the isomeric selectivity and for the differences in activities between enantiomers, and also gave support to the tentatively assigned absolute configurations of the isolated stereoisomers.

Furthermore, our findings have validated the β-lactam ring as a novel zinc-binding group, which is worthy of further investigation and optimization in the design of new HDAC8-selective inhibitors.

**Experimental Section**

**Chemical synthesis**

All reactions were performed under N₂. Commercial reagents were used as received without additional purification. Anhydrous solvents (CH₂CN, CH₂Cl₂, THF) were obtained commercially. ¹H and ¹³C NMR values were recorded on an INOVA 400, Varian INOVA 300 or a GEMINI 200 instrument with a 5 mm probe. All chemical shifts (δ) are quoted in ppm relative to residual solvent signals. Coupling constants (J) are given in Hz. FT-IR were recorded on a Nicolet 380; samples were prepared as films between NaCl plates and the wavenumbers are reported in cm⁻¹. TLC was carried out using Merck 60 F₂₅₄ plates and column chromatography was performed on Merck silica gel 200–300 mesh. GC-MS: Agilent Technologies, column HPS 5% Ph-Me. Silicon MS: Agilent Technologies MSD1100 single-quadrupole mass spectrometer, EI voltage 70 eV, gradient from 50—280°C over 30 min. HPLC-MS, HPLC: Agilent Technologies HP1100, column ZORBAX-Eclipse XDB-C8 Agilent Technologies, mobile phase: H₂O/CH₂CN, gradient from 30—80% of CH₂CN in 8 min, 80% of CH₂CN until 25 min, 0.4 mL/min. MS: Agilent Technologies MSD1100 single-quadrupole mass spectrometer, full-scan mode from m/z 50—2600, scan time 0.1 s in positive ion mode, ESI spray voltage 4500 V, nitrogen gas 35 psi, drying gas flow 11.5 mL min⁻¹, fragmentor voltage 20 V. HRMS analysis were recorded on a MAT 95 XP Thermo Finnigan.

β-Lactam 10 was prepared from commercially available 4-acetoxyazetidin-2-one following a known procedure. Product 11 is known, but was prepared following a modified procedure. Product 13 was prepared following the procedure reported in Reference [32]. Product 14 was prepared following the procedure reported in Reference [33]. Product 15 is known. Product 17 was prepared following the procedure reported in Reference [35]. Product 18 is known. Compound 23 is known, but was prepared following the procedure reported in Reference [38]. Further details on the preparation of compounds 11, 14, and 15 can be found in the Supporting Information, as can full spectral data on intermediates.
tion catalyst (29 mg, 5 mol%). After 12 h at reflux, the reaction was stopped by removal of the catalyst and the filtrate was concentrated in vacuo. Flash chromatography (cyclohexane/EtOAc, 40:60); 1H NMR (300 MHz, CD_jacetone, 22 °C): δ = 2.22–2.45 (m, 6H), 2.93 (dd, J = 2.4, J = 5.4 Hz, 1H), 3.14–3.22 (m, 1H), 2.37 (dd, J = 5.1, J = 5.4 Hz, 1H), 5.50–5.66 (m, 2H), 6.80 (br s, 1H), 7.0–7.05 (m, 1H), 7.24–7.30 (m, 2H), 7.6–7.67 (m, 2H), 9.12 ppm (br s, 1H); 13C NMR (75 MHz, CD_jacetone, 24 °C): δ = 29.3, 32.5, 37.8, 41.1, 51.9, 120.1, 124.0, 128.1, 130.1, 132.1, 140.6, 170.9, 171.4 ppm; IR (film): ν = 3301, 2923, 1736, 1664 cm⁻¹; HPLC-MS (ESI): R_t = 2.2 min, m/z: 259 [M + H]⁺; 3320, 2920, 2850, 1750 cm⁻¹; 3b [2M + Na]⁺; HRMS (EI): m/z [M⁺] calculated for C_19H_22N_2O_5: 258.1368, found: 258.1371.

On selected fractions enriched with the Z isomer, separation of the four stereoisomers using semi-preparative HPLC (Daicel Chiralcel OD, 0.46 cm ɔ × 25 cm; 0.5 mL min⁻¹; n-hexane/PrOH, 85:15) gave: 2c(Z) (R_t = 36.6 min); 2d(Z) (R_t = 38.9 min); 2a(E) (R_t = 40.8 min); 2b(E) (R_t = 44.0 min).

6-(2-oxo-azetidin-3-yl)hexanoic acid phenylamide (4): Pd-C (10% w/w) (2.1 mg, 0.2 equiv) was added to a solution of 2 (10.5 mg, 0.04 mmol) in THF (1.5 mL) and MeOH (1.5 mL) and the mixture was stirred under H_2 (8 bar). After full conversion, the reaction mixture was filtered and concentrated in vacuo to afford 4 as a colorless oil (10 mg, quantitative yield); R_t = 0.20 (cyclohexane/Hexane, 15:80); 1H NMR (300 MHz, CD_jacetone, 22 °C): δ = 1.33–1.50 (m, 4H), 1.59–1.74 (m, 4H), 2.36 (t, J = 7.2 Hz, 2H), 2.94 (dd, J = 2.7, J = 5.4 Hz, 1H), 3.11–3.19 (m, 1H), 3.35 (dd, J = 5.4, J = 5.4 Hz, 1H), 6.83 (br s, 1H), 6.99–7.05 (m, 1H), 7.23–7.30 (m, 2H), 7.65–7.68 (m, 2H, 9.13 ppm (br s, 1H); 13C NMR (50 MHz, CD_jacetone, 24 °C): δ = 26.2, 27.6, 29.8, 29.9, 37.7, 42.0, 52.4, 120.1, 124.0, 129.5, 140.7, 171.5, 172.0 ppm; IR (film): ν = 3301, 2906, 2926, 1736, 1664 cm⁻¹; HPLC-MS (ESI): R_t = 7.0 min, m/z: 261 [M + H]⁺, 543 [2M + Na]⁺; HRMS (EI): m/z [M⁺] calculated for C_19H_20N_2O_5: 260.1525, found: 260.1527.

3- Allyl-1-methylsulfonyl-azetidin-2-one (16): A solution of 15 (80 mg, 0.7 mmol) in THF (4 mL) at 78 °C was first treated with LiHMDS (0.8 mmol, 1 mL in THF) and then S-methyl methanethiolate (0.2 mL, 1.8 mmol). The solution was warmed to RT and stirred for 3 h. The reaction was quenched with aq NaHCO_3 and extracted with EtOAc. The organic extracts were dried (Na_2SO_4), filtered and concentrated in vacuo. Flash chromatography (cyclohexane/EtOAc, 80:20) gave 16 as a pale yellow oil (70 mg, 62% yield); R_t = 0.75 (cyclohexane/EtOAc, 50:50).

(E)-6-(1-methylsulfonyl-2-oxo-azetidin-3-yl)hex-4-enoic acid phenylamide (3): A solution of 16 (63 mg, 0.44 mmol) and N-phenylpent-4-ename (176 mg, 1 mmol) in CH_2Cl_2 (2 mL) was degassed using the freeze-pump-thaw procedure and treated with Grubbs 2nd-generation catalyst (17 mg, 5 mol%). After 4 h at reflux, the reaction was filtered to remove the catalyst, and the filtrate was concentrated in vacuo. Flash chromatography (cyclohexane/EtOAc, 70:30) gave 3 as a colorless oil (45 mg, 37%); R_t = 0.35 (cyclohexane/EtOAc, 50:50); 1H NMR (200 MHz, CDCl_3, 22 °C): δ = 2.22–2.46 (m, 6H), 2.37 (s, 3H), 3.10 (dd, J = 3.0 Hz, J = 5.4 Hz, 1H), 2.33–3.33 (m, 1H), 3.40 (dd, J = 5.2, J = 5.4 Hz, 1H), 5.40–5.64 (m, 2H), 7.03–7.10 (m, 1H), 7.26–7.34 (m, 2H), 7.53–7.58 (m, 2H), 8.28 ppm (br s, 1H); 13C NMR (50 MHz, CDCl_3, 22 °C): δ = 21.4, 28.5, 31.3, 30.9, 48.5, 51.3, 119.8, 123.9, 126.8, 129.1, 132.1, 139.5, 171.2, 173.4 ppm; IR (film): ν = 3220, 2922, 1736, 1655 cm⁻¹; HPLC-MS (ESI): R_t = 7.1 min, m/z: 305 [M + H]⁺, 327 [M + Na]⁺; HRMS (EI): m/z [M⁺] calculated for C_17H_18N_2O_3: 304.1245, found: 304.1244.

On selected fractions enriched with the Z isomer, separation of the four stereoisomers using semi-preparative HPLC (Daicel Chiralce
tored by TLC). After 4.5 h the reaction was concentrated in vacuo. Flash chromatography (cyclohexane/ETOAc, 90:10) gave 20 as a pale yellow oil (36 mg, 96%); Rf = 0.60 (cyclohexane/ETOAc, 70:30).

6-(Benzyloxy-2-oxo-azetidin-3-yl)hex-4-en-4-ic acid phenylamide (21): A solution of 20 (26 mg, 0.12 mmol) and N-phenylpent-4-enamide (52 mg, 0.30 mmol) in CH2Cl2 (2 mL) was degassed using the freeze-pump-thaw procedure and treated with Grubbs 2nd-generation catalyst (5 mg, 5 mol%). The reaction mixture was refluxed and monitored by TLC. After 20 h at reflux, the reaction was cooled, filtered and concentrated in vacuo. Flash chromatography (cyclohexane/ETOAc, 80:20) gave 21 as a colorless oil (20 mg, 44%); Rf = 0.30 (cyclohexane/ETOAc, 70:30).

6-(1-Hydroxy-2-oxo-azetidin-3-yl)hexanoic acid phenylamide (5): Pd-C (10% w/w) (3.0 mg, 20%) was added to a solution of 21 (30.0 mg, 0.08 mmol) in THF and MeOH (2.5 mL, 4:1) and the mixture was stirred under H2 (1 bar). The reaction was monitored by TLC. After completion, the reaction was filtered and concentrated in vacuo. Flash chromatography (cyclohexane/ETOAc, 70:30) gave 5 as a colorless oil (22 mg, quantitative yield); Rf = 0.40 (cyclohexane/ETOAc, 50:50); 1H NMR (300 MHz, D2O, 22°C, 22 C): δ = 1.27–1.72 (m, 8 H), 2.35 (t, 2 H, J = 7.5 Hz), 2.83–2.88 (m, 1 H), 3.17 (dd, 1 H, J = 2.4 Hz, J = 4.5 Hz), 3.58 (dd, 1 H, J = 4.5 Hz, J = 5.1 Hz), 6.98–7.04 (m, 1 H), 7.23–7.29 (m, 2 H), 7.63–7.66 (m, 2 H), 9.02 (br s, 1 H); 9.09 ppm (br s, 1 H); 13C NMR (75 MHz, D2O, 22°C, 24 C): δ = 26.0, 27.5, 27.7, 29.7, 37.5, 45.6, 52.6, 123.9, 124.9, 140.5, 167.1 ppm; IR (film): ν = 3450, 1740, 1710, 1660 cm−1; HPLC-MS (ESI): Rf = 4.8 min, m/z: 277 [M+H]+, 575 [2M+Na]+; HRMS (EI): m/z [M]+ calcd for C16H15N2O2: 276.1474, found: 276.1474.

Separation of the two enantiomers using semi-preparative HPLC (Daicel Chiralpak IC, 0.46 cm 2 × 25 cm; 0.6 mL/min 2; n-hexane/ (iPr)OH, 70:30) gave 5a (Rf = 20.5 min); Rf = 21.4 min (n-hexane/ (iPr)OH, 70:30).

2-Hydroxymethyl-7-phenylcarbamoylhept-4-en-4-ic acid methyl ester (22): A solution of 18 (60 mg, 0.42 mmol) and N-phenylpent-4-enamide (182 mg, 1.04 mmol) in CH2Cl2 (2 mL) was degassed using the freeze-pump-thaw procedure and treated with Grubbs 2nd-generation catalyst (5 mg, 5 mol%). The reaction mixture was refluxed and monitored by TLC until disappearance of the starting material. The reaction was cooled, filtered to remove the catalyst, and concentrated in vacuo. Flash chromatography (cyclohexane/ETOAc, 75:25) gave 22 as a colorless oil (58 mg, 47%); Rf = 0.30 (cyclohexane/ETOAc, 60:40).

2-[2-Hydroxymethyl-7-phenylcarbamoylhept-4-enylamino]phenoxy)carbamic acid benzyl ester (24): nBuLi (0.75 mmol, 1.6 M in hexane) was added dropwise to an ice-cold solution of hexamethyldisilazane (140 μL, 0.67 mmol) in THF (1 mL). The ice bath was removed and the reaction was stirred at RT for 30 min. The mixture was added dropwise via cannula to a suspension of (2-aminoethyl)carbamic acid benzyl ester (24 mg, 0.10 mmol) in THF (0.8 mL) at −78°C. Finally, a solution of 22 (28 mg, 0.10 mmol) in THF (1.5 mL) was added dropwise to the mixture at −78°C. The mixture was allowed to slowly reach RT and monitored by TLC until disappearance of the starting material. The reaction was quenched with aq NH4Cl, extracted with ETOAc, and the organic extracts were dried (Na2SO4), filtered and concentrated in vacuo. Flash chromatography (cyclohexane/ETOAc, 50:50) gave 24 as a pale yellow oil (104 mg, 67%); Rf = 0.35 (cyclohexane/ETOAc, 30:70).

2-[2-Oxo-3-(5-phenylcarbamoylpent-2-ynyl)azetidin-1-yl]phenyl)carbamic acid benzyl ester (25): A solution of 24 (100 mg, 0.20 mmol) in THF (11 mL) was treated with PPh3 (58 mg, 0.22 mmol) and DIAD (43 μL, 0.22 mmol) and stirred at RT (monitored by TLC). After full conversion, the mixture was concentrated in vacuo. Flash chromatography (cyclohexane/ETOAc, 50:50) gave 25 as a pale yellow oil (75 mg, 77%); Rf = 0.70 (cyclohexane/ETOAc, 30:70).

Toluene-4-thiosulfonic acid 5-biphenyl-4-ymethyl ester (26): 4-Phenylbenzyl chloride (101 mg, 0.55 mmol) was added to a solution of potassium thiosalicylate (113 mg, 0.55 mmol) in DMF (2 mL). After 48 h, the reaction was quenched with aq HCl (0.1 M) and extracted with EtO, the organic extracts were dried (Na2SO4), filtered and concentrated in vacuo. Flash chromatography (cyclohexane/ETOAc, 90:10) gave 26 as a colorless oil (150 mg, 85% yield); Rf = 0.40 (cyclohexane/ETOAc, 90:10).

1-(Biphenyl-4-ylmethylsulfonyl)azetidin-2-one (9): A solution of azetidin-2-one (14 mg, 0.22 mmol) in THF (5 mL) at −78°C was treated first with LiHMDS (0.22 mmol, 1.1 M in THF) and then S-(4-phenyl)benzyl-4-methylbenzenesulfonothioate (90 mg, 0.25 mmol). The solution was allowed to warm to RT. After 2 h the reaction was cooled to 0°C and quenched with aq NH4Cl. The mixture was extracted with ETOAc, the organic extracts were dried (Na2SO4), filtered and concentrated in vacuo. Flash chromatography (cyclohexane/ETOAc, 80:20) gave 9 as a yellow oil (45 mg, 84% yield); Rf = 0.44 (cyclohexane/ETOAc, 80:20); 1H NMR (300 MHz, CDCl3, 22 C): δ = 2.87–2.95 (m, 4 H), 3.94–3.99 (m, 2 H), 7.28–7.56 ppm (m, 9 H); 13C NMR (75 MHz, CDCl3, 24 C): δ = 38.6, 42.5, 123.9, 126.9, 127.3, 128.8, 132.7, 135.1, 140.3, 140.5, 170.9 ppm; IR (film): ν = 3050, 3000, 1757 cm−1; HPLC-MS (ESI): Rf = 9.5 min, m/z: 270 [M+H]+, 287 [M+H2O]+, 292 [M+Na]+, 561 [2M+Na]+; HRMS (EI): m/z [M]+ calcd for C16H14N2OS: 269.0874, found: 269.0873.

Biological assay

The enzyme inhibition assay was carried out as previously described.[21] Briefly, compounds were tested in 10-dose IC50 mode in duplicate with threefold serial dilutions starting at 50 μM against human HDAC enzymes (1–11). A fluorogenic peptide was used as the substrate, consisting of a fluorogenic moiety bound to a specific p53 fragment (residues 379–392, ArgHisLysLys(Ac)), which in- cludes an acetylated lysine side chain. Upon deacylation of the substrate, the fluorophore was released giving rise to fluorescence, which was detected using a fluorimeter, and the IC50 values of the test compounds were determined by analyzing dose-response inhibition curves.
Computational analysis

Determination of the geometry of metal–ligand interaction: The zinc-binding group, represented by 1-methylsulfanyl-azetidin-2-one, was complexed to Zn$^{2+}$ in a bidentate geometry. The conformation was minimized using semi-empirical PM3 method and then an ab initio Hartree–Fock 3-21G* single-point calculation was applied to determine the energy of the complex (SPARTAN/04; Wavefunction, Inc., http://www.wavefun.com).

Homology modeling of HDAC2 isoform: We built a 3D model of HDAC2 using HDAC8 as the main template (PDB code: 1T67) and HDLP as the template for L1 and L7 loops (PDB code: 1C3S). MS-344 was used as an internal ligand during the modeling phase (~10 Å from it) and as external ligand during the validation phase, obtaining an RMSD value of 1.0 between the co-crystallized and docked solution. Homology modeling was performed by Prime 1.5 (Schrödinger LLC, Portland, USA) and refinement using Macromodel (version 9.1; Schrödinger). Docking procedure was performed by Glide (version 4.0; Schrödinger).

Molecular dynamics and minimization: Molecular dynamics of each HDAC isoform (HDAC2, homology model; HDAC7, PDB code: 3CO; HDAC8, PDB code: 1T67) with the ligand 3c was carried out at 300 K with time step of 1.5 fs. Amino acids side chains within a shell of 5 Å from the ligand were taken unconstrained, while the backbone was frozen; the Zn$^{2+}$–water distance was constrained to 2.0 ± 0.2 Å. The system was equilibrated for a period of 100 ps, followed by a production run of 1 ns, using Macromodel (version 9.1; Schrödinger). Then, 100 solutions were saved and minimized (TNGC; 2000 steps up to 0.1 gradient threshold). ΔG_{binding} was calculated using the MM-GBSA method, performed by Prime 1.5 (Schrödinger).

Docking procedure: Eight compounds, owing to three different series, were docked on HDAC8 using the refined complex with 3c. Docking procedure was performed on Glide (version 4.0; Schrödinger), using SP protocol. Poses with the best G score were selected and compared in terms of ΔG_{binding}.

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