2019

Selective Inhibition Of Histone Deacetylases

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Selective Inhibition Of Histone Deacetylases

Abstract
Reversible lysine acetylation serves as a critical regulatory pathway for diverse cellular processes. As a result, the dysregulation of proteinaceous acetyl-L-lysine hydrolysis is connected to severe medical conditions including neurological disorders, immune dysfunction, and cancer. Inhibition of the enzymes responsible for catalyzing this reaction, histone deacetylases (HDACs), has demonstrated promising results as a route to clinical intervention in many of these diseases. Of the 18 known HDACs, 11 are metal-dependent enzymes that have similar mechanisms and each regulates the function of numerous protein substrates in vivo. This frustrates the design of small molecules targeting a single isozyme, meaning that modern FDA-approved HDAC inhibitors exhibit various side effects that make them less-than-optimal for broad clinical application.

This thesis describes the characterization of HDAC–inhibitor complexes by crystallography, supported by thermodynamic and enzymological measurements, focusing on a class I enzyme, HDAC8, and a class IIIb enzyme, HDAC6. Structural analysis of complexes with inhibitors exhibiting class- or isozyme-selective activity has illuminated the structural underpinnings of isozyme-selective HDAC inhibition. For instance, irreversible inhibition of class I HDACs by the epoxyketone-based inhibitor trapoxin A is due to the conformation of the epoxide group, rather than a long-presumed covalent modification in the active site. With regard to HDAC6, selective hydroxamates exhibit an unusual monodentate metal-coordination mode mediated by steric interactions at the protein surface. HDAC6 is also predisposed to be inhibited by hydroxamates over other isozymes due to a unique entropic gain associated with inhibitor binding. Finally, mercaptoacetamides serve as an alternative, non-genotoxic zinc-binding group that can exploit subtle mechanistic differences between isozymes. Taken together, these studies have constructed a framework for the design of selective HDAC inhibitors for better-targeted therapeutics.

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SELECTIVE INHIBITION OF HISTONE DEACETYLASES

Nicholas John Porter

A DISSERTATION

in

Chemistry

Presented to the Faculties of the University of Pennsylvania

in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

2019

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Dedication

For my parents, Thomas and Helen,

and my brother, Mitchell,

for their endless and unwavering support.
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The first and foremost thanks must go to my mentor throughout this process, Prof. David W. Christianson. His support and guidance have been inexhaustible and vital to my success here at Penn. His leadership, passion, and generosity with his knowledge have exemplified to me what it means to be a mentor and educator, a model which will surely serve me well wherever the future may take me. I must also thank my committee members: Prof. E. James Petersson, who provided constant encouragement to challenge myself; Prof. Elizabeth Rhoades, who offered a welcome ear when discussing new experiments; and Prof. Jeffery G. Saven, who instilled in me the value of fundamental knowledge and its context in the bigger picture. I thank my undergraduate research advisor, Prof. John Caradonna, for guiding me through my first experience with independent biochemical research and his advice over the years. I thank all of you for your time and patience.

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Finally, I have to extend the greatest of thanks to my family around the world for their constant love and support, without which none of this would have ever been possible.
ABSTRACT

SELECTIVE INHIBITION OF HISTONE DEACETYLASES

Nicholas J. Porter
Dr. David W. Christianson

Reversible lysine acetylation serves as a critical regulatory pathway for diverse cellular processes. As a result, the dysregulation of proteinaceous acetyl-L-lysine hydrolysis is connected to severe medical conditions including neurological disorders, immune dysfunction, and cancer. Inhibition of the enzymes responsible for catalyzing this reaction, histone deacetylases (HDACs), has demonstrated promising results as a route to clinical intervention in many of these diseases. Of the 18 known HDACs, 11 are metal-dependent enzymes that have similar mechanisms and each regulates the function of numerous protein substrates in vivo. This frustrates the design of small molecules targeting a single isozyme, meaning that modern FDA-approved HDAC inhibitors exhibit various side effects that make them less-than-optimal for broad clinical application.

This thesis describes the characterization of HDAC–inhibitor complexes by crystallography, supported by thermodynamic and enzymological measurements, focusing on a class I enzyme, HDAC8, and a class IIb enzyme, HDAC6. Structural analysis of complexes with inhibitors exhibiting class- or isozyme-selective activity has illuminated the structural underpinnings of isozyme-selective HDAC inhibition. For instance, irreversible inhibition of class I HDACs by the epoxyketone-based inhibitor trapoxin A is due to the conformation of the epoxide group, rather than a long-presumed covalent modification in the active site. With regard to HDAC6, selective
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# Table of Contents

Dedication .................................................................................................................. ii

Acknowledgements ..................................................................................................... iii

Abstract ...................................................................................................................... v

Table of Contents ........................................................................................................ vii

Table of Tables ............................................................................................................. xi

Table of Figures ............................................................................................................ xii

List of Abbreviations ................................................................................................... xiv

Chapter 1 | Background................................................................................................. 1

1.1 | Post-translational modifications........................................................................ 1

1.2 | The Acetylome .................................................................................................... 3

1.3 | Arginase-deacetylase fold................................................................................... 5

1.4 | Metal-dependent HDAC isozymes....................................................................... 5

1.5 | Mechanism of amide bond hydrolysis................................................................... 9

1.6 | Structural basis of substrate specificity.............................................................. 13

1.7 | Regulation by corepressors................................................................................ 16

1.8 | Inhibitor binding and isozyme selectivity........................................................... 17

1.9 | Focus of Thesis................................................................................................... 19

Chapter 2 | Class I HDAC Inhibition by Trapoxin A.............................................................. 22

2.1 | Introduction....................................................................................................... 22
2.2 | Materials & Methods ........................................................................................................25

2.2.1 – Chemical Reagents.....................................................................................................25

2.2.2 – Protein Expression and Purification........................................................................25

2.2.3 – C153S HDAC8 mutagenesis .................................................................................27

2.2.4 – Crystallization ...........................................................................................................27

2.2.5 – Crystal Structure Determination ............................................................................28

2.2.6 – Inhibitor Reversibility Dialysis Assay .....................................................................28

2.2.7 – Isothermal Titration Calorimetry ..........................................................................29

2.2.8 – Matrix-Assisted Laser Desorption/Ionization (MALDI) Mass Spectrometry ........30

2.2.9 – Tryptic Peptide LC-MS/MS .................................................................................30

2.2.10 – Liquid Chromatography-Mass Spectrometry (LC-MS) ........................................31

2.3 | Results & Discussion ....................................................................................................34

2.4 | Conclusion .......................................................................................................................52

Chapter 3 | Hydroxamate Denticity in HDAC6 Inhibition ..........................................................53

3.1 | Introduction ......................................................................................................................53

3.2 | Materials & Methods .......................................................................................................56

3.2.1 – Reagents ..................................................................................................................56

3.2.2 – Protein Preparation .................................................................................................56

3.2.3 – Crystallization ..........................................................................................................57

3.2.4 – Data Collection and Structure Determination ..........................................................58

3.3 | Results ...............................................................................................................................62

3.3.1 – HDAC6–HPB Complex .........................................................................................62

3.3.2 – HDAC6–ACY-1083 Complex ...............................................................................64
5.2.3 – Crystallization ............................................................................................................... 101
5.2.4 – Data collection and structure determination ................................................................. 102
5.2.5 – Isothermal Titration Calorimetry.................................................................................. 103
5.3 | Results and Discussion ................................................................................................... 105
  5.3.1 – X-ray crystallography .................................................................................................. 105
  5.3.2 – Isothermal Titration Calorimetry (ITC) .................................................................... 112
5.4 | Conclusions ..................................................................................................................... 117

Chapter 6 | HDAC6-Selective Inhibition by Mercaptoacetamides .............................................. 119
  6.1 | Introduction ..................................................................................................................... 119
  6.2 | Materials and Methods .................................................................................................... 122
    6.2.1 - Reagents .................................................................................................................. 122
    6.2.2 - Expression and purification of HDAC6 and HDAC8 for assay ................................. 122
    6.2.3 - Inhibitory potencies against HDAC6 and HDAC8. .................................................. 123
    6.2.4 - Expression and purification of HDAC6 for crystallization ....................................... 123
    6.2.5 - Crystallographic data collection and structure determination ............................... 125
  6.3 | Results and Discussion .................................................................................................... 127
  6.4 | Conclusion ....................................................................................................................... 134

Chapter 7 | Summary & Prospects .................................................................................................... 135

Bibliography ......................................................................................................................... 138
Table of Tables

Table 2.1 | Structural statistics for the HDAC8$_{374}$-trapoxin A complex ........................................ 33
Table 2.2 | Trapoxin A-modified sites in HDAC8 by LC-MS/MS ...................................................... 43
Table 3.1 | Structural statistics for HDAC6-inhibitor complexes....................................................... 60
Table 3.2 | Structural statistics for HDAC6-R-TSA complexes at variant pH values .................. 61
Table 4.1 | Structural statistics for HDAC6 complexes with bulky inhibitors ............................. 85
Table 4.2 | Average interatomic distances in monodentate inhibitor complexes (Å) .................. 88
Table 5.1 | Structural statistics for HDAC6 complexes with capless cyclohydroxamates ......... 104
Table 5.2 | Average interatomic distances in HDAC6-cyclohydroxamate complexes (Å) .... 107
Table 6.1 | Structural statistics for the HDAC6-MCA complex ......................................................... 125
# Table of Figures

<p>| Figure 1.1 | Common post-translation modifications | 2 |
| Figure 1.2 | Representation of the acetylome | 4 |
| Figure 1.3 | Arginase-deacetylase family of proteins | 6 |
| Figure 1.4 | Phylogenetic classes of histone deacetylases | 8 |
| Figure 1.5 | Mechanism of metal-dependent histone deacetylase 8 (HDAC8) | 10 |
| Figure 1.6 | HDAC6–HC toxin complex | 12 |
| Figure 1.7 | Substrate binding to HDAC isozymes | 14 |
| Figure 1.8 | HDAC inhibitors | 18 |
| Figure 2.1 | Cyclic peptide HDAC inhibitors | 24 |
| Figure 2.2 | HDAC8–trapoxin A complex at 1.24 Å resolution | 35 |
| Figure 2.3 | Free and enzyme-bound trapoxin A | 36 |
| Figure 2.4 | Trapoxin A and peptide substrate binding | 37 |
| Figure 2.5 | HDAC8 inhibitor reversibility assays | 40 |
| Figure 2.6 | MALDI mass spectra for HDAC8 incubated with trapoxin A | 41 |
| Figure 2.7 | Trapoxin A-modified sites in HDAC8 by LC-MS/MS | 42 |
| Figure 2.8 | Mass spectra of trapoxin A in the presence and absence of HDAC8 | 44 |
| Figure 2.9 | ITC thermograms for HDAC inhibitor binding | 46 |
| Figure 2.10 | Omit map for hydrogen atoms in HDAC8–trapoxin A complex | 47 |
| Figure 2.11 | Zinc coordination interactions in HDAC8–inhibitor complexes | 48 |
| Figure 2.12 | Epoxyketone binding modes in different classes of HDACs | 51 |
| Figure 3.1 | Selectivity of HDAC inhibitors for HDAC6 over HDAC1 | 55 |
| Figure 3.2 | HDAC6–HPB complex at 1.82 Å resolution | 63 |</p>
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3</td>
<td>HDAC6–ACY-1083 complex at 1.75 Å resolution</td>
</tr>
<tr>
<td>3.4</td>
<td>HDAC6–Ricolinostat complex at 1.70 Å resolution</td>
</tr>
<tr>
<td>3.5</td>
<td>HDAC6–R-TSA complex at 1.05 Å resolution</td>
</tr>
<tr>
<td>3.6</td>
<td>Bidentate and monodentate hydroxamate–zinc binding modes</td>
</tr>
<tr>
<td>3.7</td>
<td>Surface loops in HDACs 1, 2, 3, and 6</td>
</tr>
<tr>
<td>3.8</td>
<td>Reinterpretation of the HDAC6–Nexturastat A complex (PDB 5G0I)</td>
</tr>
<tr>
<td>4.1</td>
<td>Phenylhydroxamate-based HDAC6 inhibitors</td>
</tr>
<tr>
<td>4.2</td>
<td>Polder omit maps of bulky hydroxamates complexed with HDAC6</td>
</tr>
<tr>
<td>4.3</td>
<td>Binding of compound 1 to HDAC6 and packing in the L1 loop pocket</td>
</tr>
<tr>
<td>4.4</td>
<td>Active site constriction in HDAC3 relative to HDAC6</td>
</tr>
<tr>
<td>5.1</td>
<td>Capless HDAC6-selective inhibitors</td>
</tr>
<tr>
<td>5.2</td>
<td>Polder omit maps for capless cyclohydroxamates bound to HDAC6</td>
</tr>
<tr>
<td>5.3</td>
<td>Superposition of HPB linker with phenylhydroxamate (1)</td>
</tr>
<tr>
<td>5.4</td>
<td>Dihedral angles in conjugated aromatic hydroxamic acids</td>
</tr>
<tr>
<td>5.5</td>
<td>Selectivity of hydroxamate inhibitors for HDAC6 versus HDAC8</td>
</tr>
<tr>
<td>5.6</td>
<td>Thermodynamic values for the binding of hydroxamate inhibitors</td>
</tr>
<tr>
<td>6.1</td>
<td>Mercaptoacetamide selectivity and the hydroxamate Lossen rearrangement</td>
</tr>
<tr>
<td>6.2</td>
<td>Binding of MCA to HDAC6 catalytic domain 2</td>
</tr>
<tr>
<td>6.3</td>
<td>Mercaptoacetamide binding to HDAC6 and HDAC8</td>
</tr>
</tbody>
</table>
List of Abbreviations

ACY-1083..............................................2-((4,4-difluoro-1-phenylcyclohexyl)amino)-N-hydroxypyrimidine-5-carboxamide
AcuC ..................................................................acetoacetate utilization protein
ALS ..................................................................Advanced Light Source
AMC ..................................................................aminomethylcoumarin
APAH ................................................................. acetylpolyamine amidohydrolase
APS ..................................................................Advanced Photon Source
CCP4 ................................................................. Collaborative Computational Project Number 4
CD1 ................................................................. histone deacetylase 6 catalytic domain 1
CD2 ................................................................. histone deacetylase 6 catalytic domain 2
CdLS ................................................................. Cornelia de Lange syndrome
DMSO ............................................................... dimethyl sulfoxide
deoxyribonucleic acid
FDA ................................................................. Food and Drug Administration
FIGase ............................................................. formiminoglutamase
FOXP3 .............................................................. forkhead box protein 3
HDAC ............................................................... histone deacetylase
HDACi .............................................................. histone deacetylase inhibitor
HEPES ............................................................ 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
HPB ..................................................................N-hydroxy-4-[(N(2-hydroxyethyl)-2-phenylacetamido)methyl]-benzamide]
HPOB ..................................................................N-hydroxy-4-(2-[(2-hydroxyethyl)(phenyl)amino]-2-oxoethyl)benzamide
hPro ..................................................................homoproline / pipecolic acid
IL-10 ................................................................. interleukin 10
IPTG ................................................................. isopropyl-β-thiogalactopyranoside
ITC ................................................................. isothermal titration calorimetry
L-Aoe ............................................................... (2S,9S)-2-amino-8-oxo-9,10-epoxydecanoic acid
LB ................................................................. lysogeny broth
LC-MS ............................................................. liquid chromatography-mass spectrometry
LC-MS/MS ......................................................... liquid chromatography-tandem mass spectrometry
MALDI ........................................................... matrix-assisted laser desorption/ionization
MBP................................................................. maltose binding protein
MCA......................................................... N-(5,5-dichloro-1-(indol-1-yl)pentyl)-2-mercaptoacetamide
MWCO ...................................................................molecular weight cutoff
NextA................................................................. Nexturastat A
Ni(II)-NTA ............................................................. Nickel (II) – nitrilo(triacetic acid)
NSLS2 ................................................................... National Synchrotron Lightsource-II
OD₆₀₀.......................................................................... optical density at λ=600 nm
PCR ..........................................................................polymerase chain reaction
PDB ................................................................. Protein Data Bank
PEG ........................................................................polyethylene glycol
PTM ........................................................................post-translation modification
Ricolinostat ...... 2-(diphenylamino)-N-(7-(hydroxyamino)-oxoheptyl)pyrimidine-5-carboxamide
SAHA / Vorinostat ..................................................... suberoylanilide hydroxamic acid
SMC3...........................................................................structural maintenance of chromosomes protein 3
SMRT ................................................................... silencing mediator for retinoid or thyroid-hormone receptors
SDS-PAGE ........................................................... sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SSRL ................................................................. Stanford Synchrotron Radiation Lightsource
TCEP......................................................................... tris(2-carboxyethyl)-phosphine
TEV .........................................................................tobacco etch virus
TEVP ........................................................................tobacco etch virus protease
TOF ........................................................................time-of-flight
TpxA ......................................................................... trapoxin A
TSA .............................................................................trichostatin A
UPLC-MS ............................................................ ultra-performance liquid chromatography-mass spectrometry
XDS ...........................................................................X-ray Detector Software
ΨArg ...........................................................................pseudo-arginase
ΨDAC ...........................................................................pseudo-deacetylase
1.1 Post-translational modifications

Critical biological processes are often regulated by diverse and dynamic reversible covalent modifications to protein structures that tune their function in response to changes in cellular conditions.\(^1,2\) These post-translational modifications (PTMs) include methylation, acetylation/acylation, phosphorylation, glycosylation, sumoylation, ubiquitination, and a number of other less-common modifications (Figure 1.1).\(^1-4\) These reactions alter the chemical properties of specific protein residues, resulting in either a gain or loss of function when this new functionality is introduced into a protein structure.

This is exemplified by the acetylation of lysine side chains on the N-terminal tails of histone proteins. Histone hetero-octamers wrapped in 147 base pairs of DNA form the nucleosome, the core unit of chromatin responsible for the compaction of the genome.\(^5,6\) One interaction governing nucleosome formation is charge complementarity between positively charged lysines and arginines on histone tails and the negatively charged DNA backbone.\(^7,8\) Acetylation of these lysine residues removes their positive charge, weakening this interaction and assisting in the activation of gene transcription.\(^9\) Combined with lysine and arginine methylation, phosphorylation of serine and threonine residues, and other modifications on these tails, these PTMs constitute the “histone code” that regulates dynamic transitions between transcriptionally active and silent chromatin states.\(^10,11\) These are referred to as epigenetic modifications, meaning they control gene expression without altering the genes themselves, and instead are heritable through cellular replication.\(^12,13\) However, while many PTMs were first identified on histone tails, the biological utility of these functional “switches” goes far beyond genetic regulation.\(^14\)
Figure 1.1 | Common post-translational modifications

Structures of modifying groups are shown in red. The N-terminus of histone H3.1 (blue) from a crystal structure of the human nucleosome (3AFA) is used as an example.
1.2 | The Acetylome

The reversible acetylation of lysine side chains on the surfaces of enzymes and other proteins comprises a vital regulatory strategy in myriad cellular processes such as transcription, the cell cycle, and metabolism (Figure 1.2). At present, the acetylome encompasses nearly 40,000 unique protein acetylation sites as classified in the PhosphoSitePlus database. Accordingly, acetylation rivals phosphorylation as a ubiquitous chemical modification in the regulation of protein function.

The chemistry of lysine acetylation requires “writers” – acetyl transferases that utilize acetyl-CoA as a co-substrate, and “erasers” – deacylases that catalyze the hydrolysis of acetyllysine to yield lysine and acetate. With their identification rooted in Allfrey’s pioneering discovery of histone acetylation, lysine deacetylases are historically referred to as histone deacetylases (HDACs). However, this name belies the wider function of these enzymes in processing both histone and non-histone protein substrates. Moreover, certain HDACs are not lysine deacetylases, but instead are lysine fatty-acid deacylases; others process non-protein substrates through their function as polyamine deacetylases.

The HDACs are particularly important as targets for therapeutic intervention in the treatment of cancer, neurodegenerative disease, and other disorders. To date, four different inhibitors of metal-dependent HDACs are approved in the US for cancer chemotherapy, and each inhibitor targets the active site Zn$^{2+}$ ion. Vorinostat, Belinostat, and Panobinostat contain hydroxamic acid moieties that chelate Zn$^{2+}$, and Romidepsin is a cyclic depsipeptide bearing a pendant thiol group that coordinates to Zn$^{2+}$. These inhibitors are relatively non-selective, i.e., they generally do not exhibit significant preference for the inhibition of one particular HDAC.
Figure 1.2 | Representation of the acetylome

(a) General scheme for the reversible acetylation of an L-lysine residue in protein X. (b) Representative sample of proteins (grey & colored squares) regulated by acetylation in diverse cellular processes. Adapted from Choudhary et al. Science 2009, 325, 834–840. Reprinted with permission from AAAS.
isozyme or another, so off-target side effects can result from their use. Accordingly, the development of isozyme-selective HDAC inhibitors is a priority for current drug design efforts.

1.3 | Arginase-deacetylase fold

Metal-dependent HDACs adopt an α/β fold first observed in the binuclear manganese metalloenzyme arginase, which was subsequently observed in the histone deacetylase-like protein from *Aquifex aeolicus*. This structural and evolutionary relationship was unexpected, since these two enzymes share insignificant amino acid sequence identity (15%). Even so, the Zn\(^{2+}\) binding site of the HDACs is conserved as the Mn\(^{2+}\)\(_B\) site of the arginases. Thus, metal binding stoichiometry and selectivity in the arginase-deacetylase family diverged from a common metalloenzyme ancestor.

Recent phylogenetic analysis indicates 12 clades in the arginase-deacetylase family (Figure 1.3). The arginase family also includes agmatinases, ureohydrolases, formiminoglutamases, and even pseudo-arginases that lack metal binding sites, such as the arginase-like protein of unknown function in *Trypanosoma brucei*. In addition to the HDAC isozymes, the deacetylase family also includes bacterial polyamine deacetylases, acetoin utilization proteins, and pseudo-deacetylases of unknown chemical function. The closest relationship between the arginases and deacetylases depicted in Figure 1.3 is found between human agmatinase and the pseudo-deacetylase domain of guinea pig HDAC10, which share 19% amino acid sequence identity.

1.4 | Metal-dependent HDAC isozymes

Since the discovery of the first mammalian HDAC, 11 metal-dependent HDACs have been identified and classified through phylogenetic analysis: the class I isozymes HDAC1, HDAC2, HDAC3, and HDAC8; the class IIa isozymes HDAC4, HDAC5, HDAC7, and
Figure 1.3 | Arginase–deacetylase family of proteins

Unrooted phylogenetic tree indicates 12 clades: arginases, pseudo–deacetylases (ΨArg), formiminoglutamases (FIGase) and ureohydrolases, yeast Hos3 homologues, bacterial acetylpolyamine amidohydrolases (APAH), bacterial histone deacetylases–like amidohydrolases (HDAH), class II HDACs, class I HDACs, bacterial acetoin utilization proteins (AcuC), class IV HDACs, uncharacterized protein family UPF0489, and pseudo–deacetylases (ΨDAC). Individual proteins, species, and UniProt accession numbers are listed in Supplementary Table 3 from ref. 25. Reprinted from Hai, Y. et al. Nat. Commun. 2017, 8, 15368. (Creative Commons Attribution 4.0 International License).
HDAC9; the class IIb isozymes HDAC6 and HDAC10; and the sole class IV isozyme, HDAC11 (Figure 1.4). X-ray crystal structures of metal-dependent HDACs reveal conservation of the α/β arginase-deacetylase fold: HDAC1,43,44 HDAC2,45 HDAC3,46 HDAC4,47 HDAC6,48,49 HDAC7,50 HDAC8,51,52 and HDAC10.55 Crystal structures of HDAC isozymes illuminate critical molecular features of biological function and inhibition.

Among the class I HDACs, HDAC8 is the only enzyme that exerts activity in both the nucleus and the cytosol.53 This isozyme has been shown to act upon numerous proteins in vivo that are involved in actin assembly,54,55 transcription,56 mitosis,57 and apoptotic regulation.58 However, analysis of the acetylome following the selective inhibition of HDAC8 reveals even more potential substrates involved in these and other processes including splicing, protein folding, and other enzymes that mediate post-translational modifications.59 The origin of this broad substrate profile of HDAC8 remains poorly understood. The most well-characterized substrate is structural maintenance of chromosomes protein 3 (SMC3), which is involved in the formation of the cohesion complex during mitosis.57 Single point mutations to HDAC8 can perturb this enzyme-substrate interaction to cause Cornelia de Lange syndrome (CdLS), a congenital malformation disorder.60-62

The class IIb enzyme HDAC6 plays a role in cytoskeleton maintenance,63 cellular trafficking,64 and misfolded protein response.65 It is the only HDAC enzyme bearing two separate lysine deacetylase domains, one of which primarily acts upon acetylated α-tubulin while the biological function of the other is currently unknown.66,67 However, recent studies have revealed that this domain of unknown function is capable of deacetylating C-terminal acetyllysine in vitro.48 The loss of HDAC6 function results in neuronal resistance to amyloid-β-mediated blockage of mitochondrial trafficking, so HDAC6 inhibition is thought to be a viable route to the treatment of
Histone deacetylases subdivided into phylogenetic classes with representations of protein localization, size, deficiency phenotype, and location of the lysine deacetylase (KDAC), polyamine deacetylase (PDAC), pseudo-deacetylase (ΨDAC), or lysine deacylase (KDAX) domain(s). The number of residues in the human proteins is shown to the lower right of each enzyme’s domain architecture. The class III sirtuins bear an orthogonal fold and mechanism and are not within the scope of this work. The class I HDAC8 and class IIb HDAC6 enzymes are highlighted and serve as the focus of this thesis.
neurological disorders.\textsuperscript{68,69} The requirement of HDAC6 activity for oncogenic tumorigenesis also indicates that its inhibition may provide a viable route to clinical intervention in certain cancers.\textsuperscript{70}

1.5 | Mechanism of amide bond hydrolysis

A catalytic mechanism for the hydrolysis of acetyllysine by a metal-dependent deacetylase was first proposed with the crystal structure determination of the bacterial histone deacetylase-like protein.\textsuperscript{35} Subsequent studies refined this mechanistic proposal, based in large part on structural and enzymological studies of HDAC8 (Figure 1.5). In the precatalytic Michaelis complex, the scissile carbonyl group of acetyllysine coordinates to Zn\textsuperscript{2+} and accepts a hydrogen bond from the phenol oxygen of Y306. A zinc-carbonyl interaction in the Michaelis complex is consistent with the metal-dependent variation of $K_M$ for HDAC8 against assay substrates when Fe\textsuperscript{2+} and Co\textsuperscript{2+} are substituted for Zn\textsuperscript{2+},\textsuperscript{71} as well as the crystal structures of intact substrates bound to the Y306F and H143A HDAC8 variants.\textsuperscript{72,73} Y306 may undergo induced-fit conformational changes between “out” and “in” orientations to accommodate substrate binding, as indicated by computational and experimental studies of HDAC8 and related deacetylases.\textsuperscript{47,61,74,75}

Both metal coordination and hydrogen bond interactions are required to fully activate the acetyllysine carbonyl group for nucleophilic attack by a Zn\textsuperscript{2+}-bound water molecule. Enzymological studies of wild-type HDAC8 and site-specific variants indicate that H143 serves as a general base to assist Zn\textsuperscript{2+} in activating the nucleophilic water molecule; subsequently, H143 serves as a general acid to protonate the leaving amino group, thereby enabling the collapse of the tetrahedral intermediate to yield lysine and acetate.\textsuperscript{76,77} The imidazolium side chain of H142 serves as an electrostatic catalyst, remaining protonated throughout the catalytic cycle. Both H142 and H143 hydrogen bond with the Zn\textsuperscript{2+}-bound water molecule, thereby ensuring its optimal orientation for nucleophilic attack. In class I isozymes, product acetate may depart through a “backdoor” tunnel
Enzymological and structural measurements are consistent with H143 serving as a single general base-general acid, while H142 remains in the positively charged imidazolium state to serve as an electrostatic catalyst. However, hydrogen bond differences for the tandem histidine pair in other HDAC isozymes may allow for separate general base-general acid functions in these histidine residues. Structural evidence suggests that, in HDAC6, H573 serves as a general base and H574 serves as a general acid.48
during dissociation of the enzyme–product complex.\textsuperscript{78} This tunnel may also accommodate the fatty acid product in the weak fatty acid deacylase activity measured for HDAC8.\textsuperscript{79}

Binding interactions of the tetrahedral intermediate and its flanking transition states have been visualized through the study of reactive substrate analogues that, upon binding in active site, undergo a chemical reaction that mimics the first step of catalysis. Specifically, consider the cyclic tetrapeptide HC toxin (Figure 1.6), a naturally occurring inhibitor of HDACs from \textit{Helminthosporium carbonum}.\textsuperscript{80,81} HC toxin contains a non-proteinogenic amino acid bearing an \(\alpha,\beta\)-epoxyketone amino acid, \((2S,9S)-2\text{-amino-8-oxo-9,10-epoxydecanoic acid (L-Aoe)).\) The ketone carbonyl group of the L-Aoe side chain is isosteric with the amide carbonyl group of acetyl-L-lysine, the HDAC substrate. The crystal structure of catalytic domain 2 from \textit{Danio rerio} (zebrafish) HDAC6 in complex with HC toxin shows that the ketone carbonyl undergoes nucleophilic attack to yield a tetrahedral gem-diolate that mimics the tetrahedral intermediate in catalysis.\textsuperscript{48} The presumed oxyanion of the gem-diolate coordinates to \(\text{Zn}^{2+}\) and accepts a hydrogen bond from \(\text{Y}_{745}\), and the hydroxyl group of the gem-diolate forms hydrogen bonds with \(\text{H}_{573}\) and \(\text{H}_{574}\) (Figure 1.6).

Intriguingly, cocrystallization of H574A HDAC6 with an acetyllysine-containing substrate yields an electron density map showing tetrahedral instead of planar electron density for the scissile amide moiety, suggesting that the actual tetrahedral intermediate is trapped in the active site.\textsuperscript{48} This suggests that the intact histidine, \(\text{H}_{573}\), serves as the general base in catalysis, while \(\text{H}_{574}\) serves only as a general acid, rather than as both base and acid as observed for the equivalent residue, \(\text{H}_{143}\), in HDAC8. Since the tetrahedral intermediate cannot collapse without a proton donor to the leaving amino group, the loss of general acid \(\text{H}_{574}\) results in the dead-end formation of the tetrahedral intermediate in much the same way as the tetrahedral gem-diolate of HC Toxin.
Polder omit map for HDAC toxin bound to catalytic domain 2 of *Danio rerio* HDAC6 determined at 1.73 Å resolution (PDB 5EFJ) in which the $\alpha,\beta$-epoxyketone side chain binds as a gem-diolate in the reversible enzyme-inhibitor complex. The backbone NH group of L-Aoe donates a hydrogen bond to S531, a key determinant of isozyme-substrate recognition.
is formed and stabilized in the HDAC6 active site. Crystal structures of HDAC4, HDAC6, and HDAC10 complexed with α-trifluoromethylketones similarly reveal the binding of the gem-diolate form of these inhibitors in deacetylase active sites.

As indicated in Figure 1.5, key catalytic residues in the active sites of most HDACs are the tandem histidine dyad, a catalytic tyrosine, and the Zn$^{2+}$ ion. However, in class IIa HDACs (4, 5, 7, and 9), the catalytic tyrosine is substituted with a histidine, and deacetylase activity is significantly compromised as a result. Even so, robust lysine deacetylase activity of HDAC4 and HDAC7 can be restored by histidine-to-tyrosine substitutions. The biological significance of this activity difference is unclear. The active sites of most zinc hydrolases contain an electrophile in addition to the Zn$^{2+}$ ion that helps polarize the scissile amide carbonyl, as first noted for the prototypical zinc hydrolases thermolysin and carboxypeptidase A. Possibly, the electrophilic histidine in the active sites of class IIa HDACs confers an alternative substrate specificity that is yet to be discovered.

1.6 | Structural basis of substrate specificity

X-ray crystal structures of HDAC isozymes complexed with substrates or substrate analogues reveal the molecular basis of substrate recognition. As lysine deacetylases, HDAC6 and HDAC8 catalyze the hydrolysis of acetyllysine residues contained in polypeptide and protein substrates, and the active site of each enzyme consists of a concave molecular surface surrounding a narrow cavity that accommodates the scissile acetyllysine side chain. Crystal structures of H143A HDAC8 and Y306F HDAC8 complexed with the p53-derived assay substrate Ac-Arg-His-Aly-Aly-AMC (Aly = acetyllysine, AMC = aminomethylcoumarin) show that the backbone NH groups of the scissile acetyllysine residue (boldface) and the aminomethylcoumarin moiety at the +1 position donate hydrogen bonds to the carboxylate side chain of D101 (Figure 1.7a).
Figure 1.7 | Substrate binding to HDAC isozymes

(a) Superposition of substrate complexes with Y306F HDAC8 (blue; PDB 2V5W) and H143A HDAC8 (grey; PDB 3EWF) demonstrates that the scissile acetyllysine carbonyl is activated by the Zn$^{2+}$ ion and Y306. (b) Superposition of substrate complexes with Y745F HDAC6 (dark green; PDB 5EFK) and H574A HDAC6 (light green; PDB 5EFN) reveals similar activation, but the tetrahedral intermediate is present in the complex with H574A HDAC6, suggesting that this histidine serves as only the general acid in catalysis. (c) A tetrahedral gem-diolate is observed for the trifluoromethylketone analogue of $N^8$-acetylspermidine bound to HDAC10 (PDB 5TD7) in which a 3$_{10}$-helix (purple) imposes steric constraints on the active site to confer acetylpolyamine substrate specificity.
Dual hydrogen bond interactions with D101 suggest that a proline residue at the +1 position of the substrate cannot be accommodated by HDAC8, and this has been confirmed experimentally.48 No other hydrogen bonds are observed between the concave active site surface of HDAC8 and main chain atoms for residues in the −3 to −1 positions of the bound substrate; the guanidinium group of the arginine residue at the −3 position forms hydrogen bonds with the backbone carbonyl of I94 or the carboxylate group of E148, or makes no hydrogen bonds at all in several crystal structures.72,73 The lack of strong enzyme-substrate hydrogen bond specificity presumably ensures broad specificity for flanking amino acid sequences in protein substrates. Although no structural information is available with regard to peptide substrate binding at the +2 position or beyond, there is enzymological evidence for an exosite that recognizes the basic sequence KRHR at the +4 to +7 positions of a substrate derived from the N-terminal tail of the histone H4 protein.85

In contrast with HDAC8, crystal structures of HDAC6 complexed with two different assay substrates, the α-tubulin-derived peptide Ac-Ser-Asp-Aly-AMC and the histone H4-derived peptide Ac-Arg-Gly-Aly-AMC, show that only a single hydrogen bond is made between the backbone NH group of the scissile acetyllysine residue and S531 (Figure 1.7b); the corresponding hydrogen bond is similarly observed between S531 and the NH group of the α,β-epoxyketone residue of HC Toxin.48 Accordingly, HDAC6 substrates can accommodate proline residues at the +1 position. In the HDAC6-substrate complexes, the peptide segments preceding the scissile acetyllysine residues are disordered and hence not fully modeled in the refined crystal structures.48 However, the backbone carbonyl of the aspartate or glycine residue at the −1 positions of these substrates hydrogen bonds with N530. Here, too, minimal hydrogen bonding with main chain or side chain atoms enables broad specificity for flanking amino acid sequences in protein substrates.
In contrast with HDAC6 and HDAC8, the crystal structure of HDAC10 reveals an active site cleft sterically constricted by a $3_{10}$-helix containing the highly conserved P(E,A)CE motif (Figure 1.7c). Consequently, peptide substrates cannot bind easily in the active site of HDAC10 due to the long, narrow active site cleft. Instead, the active site of HDAC10 accommodates long, slender polyamine substrates, exhibiting optimal catalytic efficiency for the deacetylation of $N^8$-acetylspermidine. A conserved gatekeeper in the HDAC10 active site, E274, ensures electrostatic recognition of the positively charged secondary amino group of $N^8$-acetylspermidine. Conserved glutamate and aspartate residues across the protein surface additionally contribute to the molecular recognition of the cationic substrate. HDAC10 appears to be the long-sought cytosolic $N^8$-acetylspermidine deacetylase for which cellular activity was discovered more than 40 years ago.

### 1.7 Regulation by corepressors

Several metal-dependent HDACs function in multi-protein regulatory complexes. For example, crystal structures of HDAC1 and HDAC3 complexed with corepressor proteins that activate catalysis reveal significant protein-protein interactions that sandwich inositol tetraphosphate at the interface. Corepressor binding buttresses the L1 loop flanking the deacetylase active site, which reduces enzyme surface dynamics. Inositol tetraphosphate interacts with a conserved arginine side chain on the L6 loop, which forms one wall of the active site. Mutation of this arginine in the HDAC3-SMRT corepressor complex results in a dramatic loss of enzyme activity, suggesting that this interaction is vital for activation of these class I HDACs.

Molecular dynamics simulations of HDAC3 reveal conformational mobility for the catalytic tyrosine in the active site, which adopts the “out” conformation in the absence of corepressor.
and the catalytically-required “in” conformation upon the binding of corepressor. Similar conformational mobility has been observed for this residue in molecular dynamics studies of HDAC8 and in X-ray crystallographic studies of a bacterial polyamine deacetylase. Conformational mobility of the catalytic tyrosine in HDAC active sites is facilitated by its location in a conserved glycine-rich loop. Conformational control of the catalytic tyrosine in the class I HDAC active site by deacetylase-activating domains of corepressor proteins represents a simple molecular strategy for the regulation of deacetylase activity in multi-protein complexes.

Notably, HDAC8 is the only class I enzyme that does not require a corepressor for optimal activity due to structural differences in the L1 and L6 loops that allow the enzyme to more readily adopt an active conformation without steric persuasion from a corepressor protein. Even so, active site loops in HDAC8 can adopt alternative conformations even as they accommodate derivatives of the cyclic tetrapeptide inhibitor Largazole. Inhibition of HDAC8 results in the upregulated acetylation of many different proteins in vivo, suggesting that the more flexible surface loops of this isozyme may allow it to recognize a variety of substrates throughout the cell.

1.8 | Inhibitor binding and isozyme selectivity

Given that HDACs are validated targets for therapeutic intervention in the treatment of cancer and other diseases, X-ray crystal structures provide valuable guidance for drug design. A key feature of an HDAC inhibitor is the functional group that coordinates to the active site Zn\(^{2+}\) ion. The hydroxamate group is most commonly utilized and is found in three out of four of the currently-approved HDAC-targeted drugs (Figure 1.8a). However, the hydroxamate group can be mutagenic due to degradation via the Lossen rearrangement, which generates a highly reactive isocyanate capable of alkylating cellular components (Figure 1.8b). This chemical reactivity can limit the utility of hydroxamate-containing drugs.
Figure 1.8 | HDAC inhibitors

(a) FDA-approved hydroxamate-based HDAC inhibitors for clinical use in cancer chemotherapy.

(b) Hydroxamate genotoxicity stems from their degradation through the Lossen rearrangement to the electrophilic isocyanate moiety. (c) Romidepsin is currently FDA-approved for cancer chemotherapy and can be activated by reduction of an intramolecular disulfide linkage. The marine natural product Largazole is structurally similar to Romidepsin and is activated by thioester hydrolysis. (d) Ideal tetrahedral coordination of the active site $\text{Zn}^{2+}$ ion by the thiolate side chain of Largazole is observed in the complex with HDAC8 (PDB 3RQD), in which the thiolate also accepts a hydrogen bond from Y306.
Macrocyclic peptides and depsipeptides comprise a fascinating class of HDAC inhibitors. These natural products contain pendant side chains that mimic the scissile acetyllysine side chain of an HDAC substrate. Romidepsin is a depsipeptide drug that, upon reduction of an intramolecular disulfide linkage, yields a side chain bearing a thiol group that coordinates to the active site $\text{Zn}^{2+}$ ion. While the crystal structure of an HDAC-Romidepsin complex has not yet been reported, the crystal structures of HDAC8 complexed with Largazole and its synthetic analogues, which are structurally similar to Romidepsin, have been reported (Figure 1.8c,d). 90,92

Other functional groups targeting $\text{Zn}^{2+}$ coordination in HDAC active sites include $\alpha$-tri-fluoromethylketones, which bind as $\text{Zn}^{2+}$-bound gem-diolates in the active sites of HDAC4,47 HDAC6,48 and HDAC10 (Figure 1.7c),25 and benzamides that chelate the $\text{Zn}^{2+}$ ion of HDAC2 through the anilide nitrogen and amide carbonyl groups.45 There are numerous opportunities for new HDAC inhibitor designs incorporating alternative metal-binding groups, such as those exemplified in a recent review on bioinorganic fragment-based drug discovery.93

1.9 | Focus of Thesis

The work presented herein focuses on the study and structural characterization of inhibitor binding to the class I enzyme HDAC8 as well as catalytic domain 2 (the primarily active domain) of the class IIb enzyme HDAC6. The ultimate goal of this work is to illuminate the chemical details underlying class- or isozyme-selective inhibition. In these studies, HDAC8 serves as a facile model for class I enzymes due to its activity in isolation (i.e. HDAC8 does not require a corepressor for activity as do HDACs 1, 2, and 3). Catalytic domain 2 from *Danio rerio* (zebrafish) HDAC6 serves as a more readily-crystallizable ortholog of the human enzyme in which all residues in and around the active site are generally conserved.
With regard to HDAC8, this thesis describes the complex with a microbial cyclic tetrapeptide inhibitor, trapoxin A, which bears the unusual \( \alpha,\beta \)-epoxyketone as the metal-binding group as found in HC toxin. This inhibitor exhibits irreversible inhibition against class I HDACs but can readily dissociate from class II enzymes. Structural analysis reveals that the origin of this irreversible inhibition lies in steric interactions of the epoxide group with a groove in the active site cavity coupled with hydration of the ketone moiety to a gem-diolate. This revises the long-standing hypothesis that irreversibility was due to the covalent modification of a nucleophile only found in the active sites of class I HDACs. The tight-binding nature of this inhibitor in the absence of covalent enzyme-inhibitor attachments invites new lines of inquiry in the design of potent molecules targeting the class I enzymes.

The majority of the presented work focuses on the characterization of the structural underpinnings of HDAC6-selective inhibition. Medicinal chemistry has identified a wide variety of HDAC6 inhibitors which exhibit 10–1000–fold selectivity, most of which are hydroxamate-based inhibitors bearing short, rigid aromatic linkers and large, sterically bulky capping groups. Intriguingly, many of these inhibitors are shown to exploit an unusual monodentate metal-coordination mode that is accessible in the shallower active site cavity of HDAC6 relative to other HDAC isozymes. This binding mode is largely controlled by steric interactions between the capping group and loops at the active site surface, especially the L1 loop. Fascinatingly, even small cyclohydroxamates lacking capping groups are selective HDAC6 inhibitors due to a favorable entropic gain upon binding to this isozyme, which is not observed for other HDACs. An entropic penalty is incurred upon binding to other isozymes for these and other hydroxamate compounds, suggesting that HDAC6 is predisposed to inhibition by such molecules. Finally, the characterization of a complex between HDAC6 and a selective mercaptoacetamide-based inhibitor reveals that this
zinc-binding group exhibits different interactions when bound to class I and class IIb enzymes. Additional hydrogen bonds are observed that suggest that this warhead is capable of targeting subtle mechanistic differences between HDAC isozymes.
Chapter 2 | Class I HDAC Inhibition by Trapoxin A


2.1 | Introduction

Macrocyclic HDAC inhibitors (HDACis) are comprised of rigid scaffolds to which a Zn$^{2+}$-coordinating functional group is attached through a linker that is the approximate length of a lysine side chain. This metal-binding group can vary; the cyclic depsipeptides romidepsin$^{94,95}$ and largazole$^{92,96}$ bear a pendant thiol group while the cyclic tetrapeptides apicidin$^{97}$ and HC toxin$^{80}$ bear ethylketone or $\alpha,\beta$-epoxyketone moieties, respectively (Figure 2.1). Notably, Romidepsin is the only non-hydroxamate HDACi approved by the Food and Drug Administration (FDA) for clinical use for the treatment of cutaneous and peripheral T-cell lymphoma.$^{98}$ As such, understanding the mode of inhibition by cyclic tetrapeptides bearing unique metal-coordination groups is of vital importance for the development of effective therapies.

Trapoxin A, first isolated from the microbial parasite *Heliconia ambiens*, is a macrocyclic tetrapeptide HDAC inhibitor with the amino acid sequence cyclo-[L-Phe–L-Phe–D-hPro–L-Aoe] (hPro = homoproline, also known as pipecolic acid; L-Aoe = (2S,9S)-2-amino-8-oxo-9,10-epoxydecanoic acid)$^{99}$. The $\alpha,\beta$-epoxyketone moiety of the L-Aoe side chain serves as the Zn$^{2+}$-coordinating group, and its ketone carbonyl group is isosteric with the scissile carbonyl of the HDAC substrate acetyl-L-lysine (Figure 2.1). Trapoxin A is proposed to act as an irreversible inhibitor of class I HDACs$^{100,101}$ and was used for the first isolation of HDAC1 from the nuclear extracts of human Jurkat T cells.$^{41}$ The epoxide moiety of the L-Aoe side chain was thought to
react with the nucleophilic side chain of an active site residue; however, a covalent enzyme–inhibitor complex was not observable by SDS-PAGE/autoradiography. Curiously, although trapoxin A was found to irreversibly inhibit HDAC1, a class I enzyme, it displayed reversible inhibition against the class IIb enzyme HDAC6. The molecular basis of these activity differences has remained unclear in the absence of structural data.

To address this gap in knowledge, we determined the first X-ray crystal structure of trapoxin A complexed with a class I histone deacetylase, HDAC8, at 1.24 Å resolution. These data indicate that the irreversible inhibition observed for trapoxin A against class I HDACs does not stem from a covalent modification in the active site. This is corroborated by isothermal titration calorimetry and mass spectrometry experiments. Instead, structural features of the metal-coordinating group and positioning of the epoxide ring within the active site binding pocket contribute to an exceptionally tight enzyme-inhibitor complex in which trapoxin A is effectively locked into the enzyme active site.
Figure 2.1 | Cyclic peptide HDAC inhibitors

Microbial cyclic peptide HDAC inhibitors bearing α,β-epoxyketone (trapoxin A & HC toxin), thiol (Romidepsin & Largazole), and ethylketone (apicidin) zinc binding groups that mimic the scissile amide linkage in HDAC substrate acetyl-L-lysine.
2.2 | Materials & Methods

2.2.1 – Chemical Reagents

Chemicals used in buffers and crystallization were purchased from Fischer, Sigma-Aldrich, or Hampton Research and used without further purification. Each polymerase chain reaction (PCR) was performed using PfuUltra High-Fidelity DNA polymerase (Agilent Technologies). Restriction enzyme Ssp1 was purchased from New England Biolabs and used according to the manufacturer’s instructions. Oligonucleotides for cloning and mutagenesis were synthesized by Integrated DNA Technologies. All DNA sequences were confirmed at the Genomics Analysis Core, Perelman School of Medicine, University of Pennsylvania. Escherichia coli strain DH5α (Invitrogen) was used for cloning and plasmid preparation. Trapoxin A and apicidin were purchased from Sigma-Aldrich at ≥98% purity. Coumarinyl peptide substrates were purchased from Enzo Life Sciences.

2.2.2 – Protein Expression and Purification

To generate the HDAC8 construct used for crystallization, the codon-optimized HDAC8 gene was amplified from the previously described HDAC8-His6-pET20b construct73 (primers: forward – 5'-TACTTCCAATCCAATGAGA-CTCTGGGCAGTCTCTG-3'; reverse – 5'-TTATCCACTTCCAATGTTATTACCTCATTTTCAGGTTGGCC). This was cloned into the pET His6 MBP TEV LIC cloning vector (1M), a gift from Scott Gradia (University of California, Berkeley; Addgene plasmid #29656), in-frame with a TEV-cleavable N-terminal His-MBP-tag (MBP; maltose binding protein) using ligation independent cloning.

The HDAC8 construct bearing a C-terminal hexahistidine tag (HDAC8His) was used for experiments except where otherwise noted. Wild-type and mutant HDAC8His expression was performed as previously described73 with minor modifications. Briefly, 50 mL starting cultures were
grown overnight in Lysogeny Broth (LB) in the presence of 100 μg/mL ampicillin. These were inoculated into 12 x 1 L M9 minimal media with 100 μg/mL ampicillin at 37°C. When the OD$_{600}$ reached ~1.0, expression was induced at 18°C for wild-type and 16°C for mutants by adding 200 μM ZnCl$_2$ (Hampton Research) and 100 μM isopropyl-β-thiogalactopyranoside. After expression for 18-21 hours, cells were pelleted via centrifugation and stored at -80°C until purification. HDAC8$_{His}$ was purified as previously described. All protein was concentrated over 10 kDa molecular weight cut-off (MWCO) centrifugal filters (Millipore) to 8-20 mg/mL. Protein concentrations were determined from the absorbance at 280 nm using the molar extinction coefficient 50,240 M$^{-1}$ cm$^{-1}$. Aliquots were flash-frozen in liquid nitrogen and stored at -80°C.

The His-MBP-TEV-HDAC8 construct, consisting of Ser-Asn followed by residues 8-374 of human HDAC8 and thus designated “HDAC8$_{374}$”, was used for crystallography. HDAC8$_{374}$ was expressed in the same manner as HDAC8$_{His}$, the only difference being that 50 μg/mL kanamycin was used in place of 100 μg/mL ampicillin. This construct was purified using a modified version of the protocol described for similar constructs of HDAC6. Briefly, cells were resuspended and lysed by sonication in loading buffer [50 mM Tris (pH 8.0), 300 mM KCl, 5% glycerol, 1 mM TCEP] and applied to amylose resin (New England Biolabs). Protein was eluted with 10 mM maltose and incubated with recombinant His-tobacco etch virus protease (TEVP) at 4°C overnight. The cleaved protein was run over Ni(II)-NTA resin (Qiagen). His-MBP and His-TEVP were eluted with 400 mM imidazole and removal of these proteins was verified using SDS-PAGE. If separation was less than satisfactory, flow-through fractions were then subjected to a second Ni(II)-NTA column. HDAC8$_{374}$-containing fractions were then concentrated by centrifugation to 5-10 mL and loaded onto a HiLoad SuperDex 200 column (GE Healthcare) in size exclusion buffer [50 mM Tris (pH 8.0), 150 mM KCl, 5% glycerol, 1 mM TCEP]. Purified
HDAC8_{374} was concentrated to 10-20 mg/mL and flash-frozen in liquid nitrogen for storage at -80°C.

The HDAC8_{374} construct appeared to exhibit somewhat lower activity than HDAC8_{His} with specific activities of 1030 ± 10 and 1530 ± 30 (nmol P)•(µmol E)^{-1}•min^{-1}, respectively. However, isothermal titration calorimetry (ITC) measurements of trapoxin A binding to both HDAC8 constructs reveal identical dissociation constants (K_d = 3 ± 1 nM) but lower N values for the same concentration of enzyme, suggesting that HDAC8_{374} may be slightly Zn^{2+} deficient following purification.

2.2.3 – C153S HDAC8_{His} Mutagenesis

The C153S mutation was introduced in the HDAC8-His_{6}-pET20b construct using Quik-change site-directed mutagenesis (Agilent Technologies). The forward primer sequence used was 5’-GCTTCTGGTTTCTCTTACCTGAACGATGCC-3’ and the reverse primer sequence was 5’-GGCATCGTTCAGGTAAGAGAAACCAGAAGC-3’. The PCR product was sequenced at the Genomics Analysis Core to confirm mutation. The mutant was expressed and purified using the same protocol outlined for HDAC8_{His} presented above. Plasmids for H142A and Y306F HDAC8_{His} were utilized as in previously reported studies.77

2.2.4 – Crystallization

The HDAC8_{374}-trapoxin A complex was cocrystallized in a sitting drop using the vapor diffusion method at 4°C. HDAC8_{374} (5 mg/mL) was incubated with 400 µM trapoxin A in size exclusion buffer with 1% dimethyl sulfoxide (DMSO) at room temperature for 1 hour. A 350 nL drop was then combined with 350 nL of precipitant solution (35% dioxane) and equilibrated against an 80 µL reservoir of precipitant solution. Large triangular prism crystals were observed.
after 3 days. Crystal were immersed in a cryoprotectant solution comprised of mother liquor supplemented with 15% ethylene glycol before being flash-cooled in liquid nitrogen.

2.2.5 – Crystal Structure Determination

X-ray diffraction data were collected at the Stanford Synchrotron Radiation Lightsource (SSRL), beamline 9-2. Data reduction and integration were carried out using iMosflm\(^{103}\) and scaled using Aimless in the CCP4 suite of programs.\(^{104}\) Molecular replacement was executed using the atomic coordinates of H143A HDAC8 (PDB ID: 3EWF)\(^{73}\) without ligands as a search model in Phaser.\(^{105}\) Coot was used to build and adjust the model in the electron density map, and refinement was performed with PHENIX.\(^{106,107}\) Trapoxin A, dioxane, ethylene glycol, and water molecules were added in later stages of refinement. Occasional spurious electron density peaks were present that could not be unambiguously modeled by solvent or other additives present during crystallization, so these were left uninterpreted. The quality of the final model was evaluated with MolProbity and PROCHECK.\(^{108,109}\) Multiple conformations were observed for E23, S43, V82, C102, T105, D147, E148, S150, L55, S190, M196, S204, S215, L235, K239, Q242, S246, C275, M279, S328, D333, T349, S351, and C352. Electron density was ambiguous or absent for the D87-I94 segment in the L2 loop as well as part or all of the side chains of L14, V15, K58, K60, and E106, so these were omitted from the final model. Crystallographic and refinement statistics are presented in Table 2.1.

2.2.6 – Inhibitor Reversibility Dialysis Assay

To assess the reversibility of cyclic tetrapeptide inhibition, 300 µL samples were prepared of 10 µM HDAC8\(^{\text{His}}\) with no inhibitor, 100 µM trapoxin A, or 100 µM apicidin in size exclusion buffer supplemented with 0.25% DMSO. These were incubated at room temperature for 1 hour and then transferred into 10 kDa MWCO Slide-a-Lyzer\textsuperscript{TM} cassettes (Thermo Scientific) and
dialyzed against 3 L of size exclusion buffer at 4°C. After 6 hours, 100 µL was extracted from each
cassette and the remaining 200 µL was dialyzed against 2 L of fresh size exclusion buffer overnight.
After the initial 1 hour incubation, as well as the first and second round of dialysis, a standard
discontinuous coupled fluorogenic assay was run to assess enzyme activity using the Fluor de Lys®
HDAC8 tetrapeptide assay substrate Ac-Arg-His-Lys(Ac)-Lys-(Ac)-aminomethylcoumarin (AMC) (BML-KI178-0005; Enzo Life Sciences). Lysine deacetylation by HDAC8 was meas-
ured by cleavage of the amide bond with the AMC group by trypsin, resulting in red-shifted
fluorescence. The signal was then fit to a standard curve to quantify product concentration.

Assays were performed in triplicate at room temperature. HDAC8_His was diluted to 1 uM
in size exclusion buffer and the assay substrate was diluted to 300 µM in activity assay buffer [25
mM Tris (pH 8.2), 137 mM NaCl, 2.7 mM KCl, and 1 mM MgCl₂]. Then, 25 µL of enzyme
and 25 µL of substrate were mixed and incubated at room temperature in the dark for 30 min.
The reaction was quenched by the addition of 50 µL of assay buffer containing trypsin and 200
µM suberoylanilide hydroxamic acid (SAHA; Cayman Chemical), a pan-HDAC inhibitor. Fol-
lowing 30 min of development in the dark, fluorescence was measured using a Tecan Infinite
M1000Pro plate reader (λ_ex = 360 nm, λ_em = 460 nm). Specific activity assays of wild-type
HDAC8_His and HDAC8374 were performed in the same manner and processed using a standard
curve generated by correlating known product concentrations with the signal following trypsin
digestion. Data were averaged and plotted using GraphPad Prism.

2.2.7 – Isothermal Titration Calorimetry

Experiments were performed with wild-type and Y306F HDAC8_His, as well as wild-type
HDAC8374, for both trapoxin A and apicidin while H142A and C153S HDAC8_His were studied
with trapoxin A only. Heat curves were measured using a MicroCal iTC200 isothermal titration
calorimeter (Malvern). For trapoxin A, 200 µM inhibitor was titrated against 20 µM enzymes in size exclusion buffer containing 0.5% DMSO. Due to the limited solubility of apicidin in aqueous media, 100 µM inhibitor was titrated against 10 µM enzymes in size exclusion buffer with 5% DMSO. Forty 1-µL injections were made over 80 min except for the titration of apicidin against Y306F HDAC8\textsubscript{His}. This experiment used twenty 2-µL injections over 1 hour to increase the observed heat per injection. Integration, curve fitting, and figure generation were performed using Origin (OriginLab, Northampton, MA).

**2.2.8 – Matrix-Assisted Laser Desorption/Ionization (MALDI) Mass Spectrometry**

MALDI mass spectrometry was used to verify the modification state of a sample of 50 µM HDAC8\textsubscript{His} incubated with 500 µM trapoxin A at 37°C for 18 hours prior to its submission for LC-MS/MS analysis. After incubation, 2 µL were removed and mixed with 10 µL of saturated sinapic acid (Sigma-Aldrich) in 50:50 acetonitrile:0.1% aqueous trifluoroacetic acid and then a 2 µL sample of the resulting solution was transferred to a 384-spot steel MALDI target plate (Bruker). Once the matrix had crystallized and the spots were dry, mass spectra were obtained on a Bruker Ultraflex III TOF/TOF mass spectrometer with 2000 shots per spot at 50% laser power. Data were processed in flexAnalysis (Bruker Daltonics) and mass spectra were plotted in GraphPad Prism.

**2.2.9 – Tryptic Peptide LC-MS/MS**

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to search for sites of modification of HDAC8 by trapoxin A, focusing on His and Cys residues since the closest potential nucleophiles to the epoxide in the HDAC8 complex were H142 and C153. Runs of LC-MS/MS were carried out on HDAC8\textsubscript{His} alone in size exclusion buffer as a control as well as samples consisting of (1) 480 µM HDAC8\textsubscript{His} with 480 µM trapoxin A, (2) 50 µM HDAC8\textsubscript{His} with
200 µM TpxA, and (3) 50 µM HDAC8_{His} with 500 µM TpxA. Samples 1 and 2 were incubated at room temperature for 1 hour while sample 3 was incubated at 37°C for 18 hours. Peaks consistent with mono- and di-labeled HDAC8_{His} with TpxA were observed for sample 3 by MALDI prior to LC-MS/MS analysis.

LC-MS/MS was carried out at the Wistar Institute Proteomics and Metabolomics Facility. Samples were reduced with 15 mM TCEP for 30 min and then alkylated with 15 mM iodoacetamide for 45 min. The reaction was quenched by incubation with 35 mM L-cysteine for 15 min prior to tryptic digestion. The samples were digested with trypsin overnight in the absence of denaturant. LC-MS/MS data were collected on a Q-HF mass spectrometer. MaxQuant 1.5.2.8 was used to search mass spectra against the UniProt E. coli database plus the sequence of HDAC8_{His} allowing for N-terminal acetylation, Met oxidation, Asn deamidation, C carbamidomethylation, and a mass addition of 602.3 Da at His or Cys residues corresponding to modification by trapoxin A. Modification by trapoxin A was observed at 6 sites in run 1, 10 sites in run 2, and 15 sites in run 3 (4 of which were His residues in the hexahistidine tag).

2.2.10 – Liquid Chromatography–Mass Spectrometry (LC–MS)

LC-MS was used to determine the observed mass of HDAC8 in the presence and absence of trapoxin A. Previously, it had been proposed that trapoxin A covalently modifies the enzyme in the folded state, but upon denaturation it is hydrolyzed from the enzyme.\textsuperscript{101} A sample of 200 µM trapoxin A was incubated in size exclusion buffer plus 0.5% DMSO for 1 hour with and without 200 µM HDAC8_{374}. A 50 µL aliquot of each sample was then diluted with 50 µL of acetonitrile, filtered via centrifugation, and submitted to LC-MS analysis. A 2 µL aliquot was then injected over a C_{18} reverse phase column with a gradient from 95:5 water:acetonitrile on a Waters Acquity
UPLC-MS. Mass spectra were processed using MassLynx software (Waters) and plotted using GraphPad Prism.
Table 2.1 | Structural statistics for the HDAC8<sub>374</sub>–trapoxin A complex

**HDAC8<sub>374</sub>–trapoxin A**

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<td><em>α, β, γ</em> (°)</td>
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**PDB accession code** | 5VI6

<sup>a</sup> Values in parentheses refer to data in the highest shell.  
<sup>b</sup> *R*<sub>merge</sub> = Σ<sub>hkl</sub>Σ<sub>i</sub>|I<sub>i,hkl</sub> − (I<sub>hkl</sub>)<sup>/</sup>Σ<sub>i</sub>I<sub>i,hkl</sub>, where (I<sub>hkl</sub>) is the average intensity calculated for reflection *hkl* from replicate measurements.  
<sup>c</sup> *R*<sub>p.i.m.</sub> = (Σ<sub>hkl</sub>(1/(N−1))<sup>1/2</sup>Σ<sub>i</sub>|I<sub>i,hkl</sub> − (I<sub>hkl</sub>)<sup>/</sup>Σ<sub>i</sub>I<sub>i,hkl</sub>, where (I<sub>hkl</sub>) is the average intensity calculated for reflection *hkl* from replicate measurements and N is the number of reflections.  
<sup>d</sup> Pearson correlation coefficient between random half-datasets.  
<sup>e</sup> *R*<sub>work</sub> = Σ|[F<sub>o</sub>] − |F<sub>c</sub>|<sup>/</sup>Σ|F<sub>o</sub>| for reflections contained in the working set. |F<sub>o</sub>| and |F<sub>c</sub>| are the observed and calculated structure factor amplitudes, respectively.  
<sup>f</sup> *R*<sub>free</sub> is calculated using the same expression for reflections contained in the test set held aside during refinement.  
<sup>g</sup> Calculated with PROCHECK<sup>109</sup>.
2.3 | Results & Discussion

The structure of the HDAC8–trapoxin A complex at 1.24 Å resolution (Figure 2.2) reveals that the conformation of the tetrapeptide backbone of trapoxin A is identical to that observed in the crystal structure of the uncomplexed inhibitor, with root-mean-square deviations (rmsd) of 0.21–0.24 Å for 16 main chain atoms of the cyclic peptide backbone; the L-Aoe side chains adopt different conformations (Figure 2.3). All peptide linkages of trapoxin A adopt the trans configuration except for the Phe-hPro linkage, which forms a cis-peptide. All three backbone NH groups donate hydrogen bonds to the side-chain carboxylate group of D101. The hydrogen bonds between D101 and the backbone NH groups of L-Aoe and the adjacent L-Phe residue are similar to those observed between D101 and linear tetrapeptide substrates bound to inactivated HDAC8 (Figure 2.4). Apart from interactions described below for the L-Aoe side chain, no other direct enzyme-inhibitor hydrogen bonds are observed. Although there are no intramolecular hydrogen bonds in trapoxin A, the side chains of its two L-Phe residues make a favorable quadrupole-quadrupole interaction with edge-to-face geometry.

Conformational changes are required in the enzyme active site to accommodate the steric bulk of the rigid peptide macrocycle in comparison with substrate binding, primarily in the L2 loop (residues G86-I108, of which D87-I94 are disordered). Relative to the structure of H143A HDAC8 complexed with a tetrapeptide substrate, the greatest change is observed for Y100, which undergoes a 116° change in side chain torsion angle χ1 (Figure 2.4). Similar conformational flexibility of Y100 accommodates the binding of the macrocyclic depsipeptide inhibitor largazole. In the HDAC8–trapoxin A complex, the conformational change of Y100 is triggered by one of the inhibitor L-Phe residues, with which one of two observed conformers makes a favorable edge-to-face interaction.
Figure 2.2 | HDAC8–trapoxin A complex at 1.24 Å resolution

(a) Stereoview of trapoxin A (orange) bound in the active site of HDAC8 (blue). The simulated annealing omit map for trapoxin A (green) is contoured at 3.0σ. Metal coordination and hydrogen bonding interactions are presented as solid and dashed black lines, respectively. (b) Close-up stereoview showing the zinc-bound gem-diolate.
Figure 2.3 | Free and enzyme-bound trapoxin A

Conformation of trapoxin A as bound to HDAC8 (orange) superimposed on the three molecules in the asymmetric unit of the uncomplexed trapoxin A crystal structure (CSD ID: TALDEP; cyan, light blue, blue). The side chain conformation of L-Aoe in the uncomplexed inhibitor differs from that observed in the enzyme-bound inhibitor.
Figure 2.4 | Trapoxin A and peptide substrate binding

Superposition of trapoxin A (orange) bound to HDAC8 (blue ribbon) and the coumarinyl peptide substrate RHK(Ac)K(Ac)-aminomethylcoumarin (green) bound to H143A HDAC8 (grey ribbon; PDB ID: 3EWF). Selected residues are labeled. Metal coordination interactions are shown as black lines, hydrogen-bonds with trapoxin A are shown as black dashes, and the D101-substrate hydrogen bonds are shown as gold dashed lines. A water-mediated hydrogen bond is observed between the L-Aoe carbonyl of trapoxin A and the imidazole side chain of H180, a metal-binding residue. Trapoxin A binding causes parts of the L2 loop to become disordered and Y100 to rotate outward. One major conformation of Y100 is observed, but residual electron density suggests the presence of another minor conformation making a favorable edge-to-face interaction with a Phe side chain of trapoxin A.
Surprisingly, the ketone carbonyl of the L-Aoe side chain undergoes nucleophilic attack by water upon binding to HDAC8, such that the inhibitory species is a gem-diolate (or perhaps a gem-diol) stabilized by Zn\(^{2+}\) coordination and three hydrogen bonds. Thus, trapoxin A mimics the binding of the tetrahedral intermediate and its flanking transition states in catalysis; the origins of high affinity are undoubtedly rooted in the fact that trapoxin A binds as an analogue of the postulated transition state. The Zn\(^{2+}\)–O1 and Zn\(^{2+}\)–O2 distances for the gem-diolate are 2.5 Å and 1.9 Å, respectively. The O1 hydroxyl group also forms hydrogen bonds with H142 and H143 (O––O separations = 2.7 Å each), and the O2 oxyanion accepts a hydrogen bond from the phenol oxygen of Y306 (O––O separation = 2.6 Å). While it is unusual to see an unactivated ketone binding as a tetrahedral gem-diolate, which exists to less than 0.2% in aqueous solution (based on the hydration of the unactivated ketone carbonyl of acetone in aqueous solution),\(^{111}\) it is notable that the L-Aoe side chain of the cyclic tetrapeptide inhibitor HC toxin (Figure 2.1) similarly undergoes nucleophilic attack in the recently-determined structure of its complex with catalytic domain 2 of HDAC6.\(^4^8\) This behavior is also reminiscent of the binding of unactivated aldehyde and ketone substrate analogues to carboxypeptidase A, which similarly bind as tetrahedral gem-diolate transition state analogues.\(^{112,113}\)

In the HDAC8–trapoxin A complex, the epoxide ring of the L-Aoe side chain is clearly intact and makes no hydrogen bond interactions with any enzyme residues or water molecules (Figure 2.2b). The closest side chains to the epoxide moiety are those of W141, H142, C153, and Y306 with interatomic separations of 3.3–3.7 Å. The epoxide moiety is believed to be required for essentially irreversible inhibitory activity against class I HDACs,\(^9^9\) based on the lack of irreversible inhibitory activity for the cyclic tetrapeptide inhibitor apicidin (Figure 2.1), which lacks an epoxide moiety. Thus, it is curious that the epoxide moiety of trapoxin A does not react with the enzyme.
However, the crystal structure reveals that although the epoxide moiety binds in the vicinity of catalytic general base H143 and highly conserved C153, neither of these potential nucleophiles is positioned or oriented for nucleophilic attack at the epoxide (Figure 2.2b).

We confirmed the irreversibility of trapoxin A inhibition by assaying HDAC8 activity following multiple rounds of dialysis of the enzyme-inhibitor complex. Regeneration of activity was not observed for HDAC8 preincubated with a 10-fold molar excess of trapoxin A, but was observed under the same conditions using apicidin (Figure 2.5). This result confirms that the epoxide moiety is required for essentially irreversible inhibition. However, this result does not prove that a covalent enzyme-inhibitor complex is formed.

To study the covalent modification of HDAC8 by trapoxin A in solution, we employed liquid chromatography-tandem mass spectrometry (LC-MS/MS) on HDAC8 preincubated for 18 hours with 10 molar equivalents of trapoxin A and subsequently digested with trypsin. Prior to digestion, mass peaks corresponding to HDAC8 covalently modified with one or two trapoxin A molecules were observed for this sample by MALDI mass spectrometry (Figure 2.6). Following trypsin digestion, LC-MS/MS analysis, and sequence analysis for residue modifications, a mass shift corresponding to the molecular weight of trapoxin A was sporadically observed for various cysteine and histidine residues located on the surface of HDAC8 (Figure 2.7; Table 2.2). Only nonspecific labeling of the enzyme was observed in the presence of excess inhibitor, with no particular preference for covalent modification in the active site. Additionally, incubation of trapoxin A for 1 hour in the presence and absence of HDAC8 followed by LC-MS analysis indicated the presence of only intact trapoxin A (i.e., with an intact epoxide ring; Figure 2.8). These results strongly suggest that trapoxin A is simply an exceptionally tight-binding, noncovalent transition state analogue inhibitor of HDAC8. As noted by Schramm and
Figure 2.5 | HDAC8 inhibitor reversibility assays

Recovered HDAC8 activity from samples incubated with DMSO, apicidin, and trapoxin A (TpxA) after no (gray), one (orange) or two (blue) rounds of dialysis against $10^4$-fold excess fresh buffer.
Figure 2.6 | MALDI mass spectra for HDAC8 incubated with trapoxin A

MALDI mass spectra of HDAC8 incubated with DMSO (black) or trapoxin A (TpxA, blue) at 37°C for 18 h prior to LC-MS/MS experiment.
Figure 2.7 | Trapoxin A-modified sites in HDAC8 by LC-MS/MS

Of the 17 modified sites, 16 are shown here because one site of modification, the hexahistidine tag, is disordered and hence not observed in the crystal structure. Modification was observed in one (cyan), two (green), or three (red) replicates. Modification percentages are shown in Table 2.2. Only H51 and C244 were modified in all trials, with total modification fractions ranging from 1.5–9.5%. The only sites with modification fractions exceeding 20% in a single trial were surface residues C352, H375, and residues in the hexahistidine tag at levels ranging 23–50%. Labeling of the tandem catalytic histidines H142 and H143, zinc ligand H180, and C153 was observed with modification fractions ranging 0.6–9.0% for any one of these residues.
Table 2.2 | Trapoxin A-modified sites in HDAC8 by LC-MS/MS

$N_{\text{obs}}$ refers to the total number of times that a residue was observed. $N_{\text{mod}}$ refers to the number of times that the residue was detected with a mass addition consistent with trapoxin A modification.

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<th>Run 2</th>
<th>Run 3</th>
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<tr>
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<td>50</td>
<td>50</td>
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<td>RT* for 1 hour</td>
<td>37˚C for 18 hours</td>
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<td>$N_{\text{mod}}$</td>
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*RT = room temperature
Figure 2.8 | Mass spectra of trapoxin A in the presence and absence of HDAC8

Normalized mass spectra of trapoxin A (TpxA) incubated in the presence (blue) and absence (red) of HDAC8. Monoisotopic mass peaks corresponding to the mass of trapoxin A plus a proton, sodium ion, water, and potassium ions are labeled. Interestingly, an additional peak is observed in mass spectra of both samples that is consistent with monohydrated trapoxin A. This could reflect the enhanced reactivity of the $\alpha,\beta$-epoxyketone carbonyl resulting in a higher percentage of the gem-diol in solution.
colleagues, transition state analogues with essentially irreversible binding behavior are feasible for enzymes that exhibit catalytic rate enhancements of $10^{10}$ or greater, which is likely the case for an amide hydrolase such as HDAC8 based on uncatalyzed amide bond hydrolysis half-lives measured in centuries by Radzicka and Wolfenden.

Isothermal titration calorimetry (ITC) measurements yield HDAC8 dissociation constants $K_d = 3 \pm 1$ nM for trapoxin A and $K_d = 250 \pm 70$ nM for apicidin (Figure 2.9), indicating 83-fold enhanced binding affinity for trapoxin A relative to apicidin. Given the structural similarity between the two cyclic tetrapeptides, and the fact that the macrocycle makes very few intermolecular interactions in the HDAC8–trapoxin A complex, it is clear that the epoxide moiety of trapoxin A is indeed responsible for tight binding to HDAC8.

ITC measurements of HDAC8 active site variants indicate that H142 is critical for trapoxin A binding just as it is important for catalysis, since the H142A mutation results in significantly weaker affinity with $K_d = 17 \pm 5$ μM – H142A HDAC8 exhibits a 233-fold reduced $k_{cat}/K_M$ value relative to wild-type HDAC8. This residue functions in catalysis with a positively charged imidazolium group that serves as an electrostatic catalyst. Electron density is observed for the Nε-H proton of H142 in the 1.24 Å electron density map (Figure 2.10), so the H142 imidazolium group must similarly stabilize the zinc-bound gem-diolate.

Although the L-Aoe side chains of trapoxin A and HC toxin bind to HDAC8 and HDAC6, respectively, as the gem-diolate, there are notable differences between the structures of each enzyme-inhibitor complex that may explain the tighter binding of these inhibitors to class I HDACs. In the class IIb HDAC6–HC toxin complex ($K_i = 350$ nM), the C–O bond of the epoxide adopts an energetically unfavorable eclipsed conformation with respect to the C–O bond of the hydroxyl group of the zinc-bound gem-diolate (Figure 2.11a); in the class I HDAC8–
Figure 2.9 | ITC thermograms for HDAC inhibitor binding

Isothermal titration calorimetry (ITC) thermograms and corresponding binding isotherms derived from integrating thermograms using the one-site model. Measurements were made for: 200 µM trapoxin A versus 20 µM wild-type, H142A, C153S, and Y306F HDAC8; and 100 µM apicidin versus 10 µM wild-type and Y306F HDAC8. The HDAC8 construct bearing the hexahistidine tag, HDAC8_His, was used for these measurements. The wild-type tagless construct, HDAC8_374, was also studied with trapoxin A and apicidin using identical conditions. Thermodynamic constants for each run are calculated from curve fitting and inlaid in each plot.
Simulated annealing omit map contoured at 2.5σ showing hydrogen atoms in the HDAC8-trapoxin A complex. The zinc ion is indicated by a gray sphere. A large electron density peak corresponds to the Ne-H atom of H142, indicating that this residue is in the positively charged imidazolium state.
Figure 2.11 | Zinc coordination interactions in HDAC8–inhibitor complexes

(a) HDAC8-trapoxin A complex, PDB 5VI6. (b) HDAC8-trichostatin A complex, PDB 1T64.
(c) HDAC8-largazole complex, PDB 3RQD.
trapoxin A complex ($K_d = 3 \text{nM}$), the C–O bond of the epoxide adopts an energetically favorable staggered conformation (Figure 2.11b). The energetically unfavorable eclipsed conformation in the HDAC6-HC toxin complex appears to be caused by the bulky P571 residue in the L3 loop, conserved in all class II HDACs – if the epoxide adopted an energetically favorable staggered conformation in the HDAC6 active site, the epoxide methylene group would clash with P571. In class I HDACs, P571 is not conserved and the active site is more open. Thus, a more favorable binding conformation is accessible to the epoxyketone moiety only in the active site of a class I HDAC.

Although the $\alpha,\beta$-epoxyketone epoxide moiety of trapoxin A remains intact in the crystal structure of its complex with HDAC8, it is notable that this novel functionality is chemically reactive in the binding of inhibitors to other enzymes. For example, the proteasome inhibitor carfilzomib contains an $\alpha,\beta$-epoxyketone that forms a covalent adduct to block proteasome function $^{116}$. The crystal structure of a similar natural product, epoxomicin, complexed with the yeast 20S proteasome indicates a multistep cyclization sequence leading to the formation of a morpholino ring between the former $\alpha,\beta$-epoxyketone of the inhibitor and the reactive N-terminal threonine residue of the proteasome subunit $^{117}$. A two-step mechanism for inhibitor binding is initiated by nucleophilic attack of the threonine hydroxyl group at the epoxyketone carbonyl followed by a $6 \text{Exo-Tet}$ ring closure reaction between the $\alpha$-amino group of threonine and the epoxide to generate the morpholino product $^{118}$.

The chemistry of inhibitor binding in this system indicates that the carbonyl group appears to be more reactive than the epoxide of the $\alpha,\beta$-epoxyketone moiety. This is consistent with reactivity trends observed in organic synthesis for various $\alpha,\beta$-epoxycarbonyl derivatives, where the carbonyl group preferentially undergoes nucleophilic addition while leaving the epoxide moiety
intact. With respect to trapoxin A, it is notable that an additional peak is observed in mass spectra consistent with gem-diol formation even in the absence of enzyme (Figure 2.8).

Finally, it is interesting to compare the structures of zinc coordination polyhedra in different HDAC8-inhibitor complexes (Figure 2.12). Only one oxygen of the gem-diolate of trapoxin A is sufficiently close for inner-sphere coordination, so the overall zinc coordination geometry is best described as 4-coordinate distorted tetrahedral. In this regard, the zinc coordination geometry approaches that observed in the HDAC8–largazole complex, which exhibits nearly perfect tetrahedral coordination through the binding of the largazole thiolate group. In contrast with these examples, the hydroxamate group of trichostatin A coordinates to zinc in bidentate fashion, so that the overall coordination geometry is 5-coordinate square pyramidal.
Figure 2.12 | Epoxyketone binding modes in different classes of HDACs

(a) Head-on view of the HC toxin gem-diol (yellow) bound to HDAC6 (red) alongside a surface contour of the HDAC6 active site cavity showing the steric interactions forcing the eclipsed orientation of the epoxyketone relative to the gem-diol. (b) Head-on view of the trapoxin A gem-diol (orange) bound to HDAC8 (blue) alongside a surface contour of the HDAC8 active site cavity showing the presence of an active site groove that accommodates the staggered orientation of the epoxyketone relative to the gem-diol.
2.4 | Conclusion

In conclusion, the work presented here demonstrates that trapoxin A is an essentially irreversible noncovalent inhibitor of HDAC8: the α,β-epoxyketone side chain of the inhibitor undergoes nucleophilic attack by zinc-bound water to bind as a tetrahedral gem-diolate transition state analogue. Along with a favorable staggered conformation of the intact epoxide moiety relative to the zinc-bound gem-diolate, these structural features contribute to an exceptionally tight enzyme-inhibitor complex effectively locked into the enzyme active site. This provides a starting point for the design of inhibitors exploiting this binding environment for the development of targeted scientific and clinical tools.
Chapter 3 | Hydroxamate Denticity in HDAC6 Inhibition

Work presented here is reprinted from Porter, N.J.; Mahendran, A.; Breslow, R.; Christianson, D.W. Proc. Natl. Acad. Sci. USA 2017, 114, 13459–13464. All crystallography was performed by N.J.P. The HDAC inhibitor HPB was prepared by A.M.

3.1 | Introduction

Among the HDAC isozymes, HDAC6 is distinct by virtue of its localization in the cytoplasm, as signaled by a serine/glutamate-rich repeat,\textsuperscript{122} as well as its biological function.\textsuperscript{123,124} Additionally, HDAC6 is the only isozyme that contains two catalytic domains, CD1 and CD2, the structures of which have recently been solved.\textsuperscript{48,49} One of these domains, CD2, catalyzes the deacetylation of K40 of $\alpha$-tubulin in the lumen of the microtubule.\textsuperscript{67,125} Consequently, inhibition of HDAC6 results in microtubule hyperacetylation and suppression of microtubule dynamics, leading to cell cycle arrest and apoptosis.\textsuperscript{125,126} HDAC6 is thus a critical target for the design of isozyme-selective inhibitors for use in cancer chemotherapy.\textsuperscript{127,128}

Significant advances have been made in the development of HDAC6-selective inhibitors, examples being Tubastatin,\textsuperscript{129} $N$-hydroxy-4-[(2-hydroxyethyl)(phenyl)amino]-2-oxoethyl)benzamide (HPOB),\textsuperscript{130} $N$-hydroxy-4-[(N(2-hydroxyethyl)-2-phenylacetamido)methyl]-benzamide (HPB),\textsuperscript{131} ACY-1215 (Ricolinostat),\textsuperscript{132} and ACY-1083\textsuperscript{133} (Figure 3.1). These inhibitors exhibit HDAC6 selectivities ranging from 12- to greater than 1000-fold relative to class I enzymes such as HDAC1. Each of these inhibitors targets Zn$^{2+}$ coordination with a hydroxamate group; HDAC6 selectivity is thought to be dictated by bulky capping or linker groups compared
with pan-HDAC inhibitors such as suberoylanilide hydroxamic acid (SAHA) or trichostatin A (TSA) (Figure 3.1).

To determine molecular features responsible for HDAC6-selective inhibition, we determined crystal structures of inhibitor complexes with the CD2 domain of *Danio rerio* (zebrafish) HDAC6, referred to simply as "HDAC6" for the remainder of this chapter. Recent structural and functional studies from the Christianson lab have demonstrated that zebrafish HDAC6 is a validated and more readily-crystallized surrogate of human HDAC6, the actual drug target. Here, crystal structures of HDAC6 complexed with the HDAC6-selective inhibitors HPB and ACY-1083 reveal a common monodentate hydroxamate-\(\text{Zn}^{2+}\) binding mode similar to that recently observed in the crystal structure of the HDAC6–HPOB complex. Thus, this unusual binding mode is a signature of selectivity for the binding of these bulky phenylhydroxamate inhibitors to HDAC6. In contrast, the crystal structure of the HDAC6 complex with Ricolinostat reveals a canonical bidentate hydroxamate-\(\text{Zn}^{2+}\) chelate complex, so the isozyme selectivity of this inhibitor is rooted solely in the interactions of its bulky capping group. Intriguingly, the 1.05 Å-resolution structure of the HDAC6 complex with the \(R\)-stereoisomer of TSA reveals two \(\text{Zn}^{2+}\) binding modes for this pan-HDAC inhibitor: the major binding mode corresponds to the canonical bidentate hydroxamate-\(\text{Zn}^{2+}\) complex observed in the previously determined 1.59 Å-resolution structure, and the minor binding mode corresponds to the unusual monodentate \(\text{Zn}^{2+}\) complex observed exclusively for HPB, HPOB, and ACY-1083.
Figure 3.1 | Selectivity of HDAC inhibitors for HDAC6 over HDAC1

Selectivity of hydroxamate-based inhibitors for inhibition of HDAC6 versus HDAC1 based on IC$_{50}$ assays. Values were obtained from: ‘Ref. 131; ‘Ref. 49; ‘Ref. 132; ‘Ref. 130; ‘Ref. 133; ‘Ref. 136.
3.2 | Materials & Methods

3.2.1 – Reagents

In general, chemicals used in buffers and crystallization were acquired from Fisher, Millipore Sigma, or Hampton Research and used without further purification. The inhibitor HPB was synthesized according to published procedures by the Breslow lab. Ricolinostat and ACY-1083 were the generous gift of Acetylon (now Bristol-Myers-Squibb).

3.2.2 – Protein Preparation

HDAC6 catalytic domain 2 from *Danio rerio* (henceforth simply "HDAC6") was recombinantly expressed using the MBP-TEV-z6CD2-pET28a(+) vector and purified as previously described with minor modifications. Briefly, HDAC6 was expressed by the *E. coli* BL21 (DE3) strain (Stratagene) in 2x YT medium under the selection of 50 mg/L kanamycin. Expression was induced by 100 µM isopropyl β-D-1-thiogalactopyranoside (IPTG; Gold Biotechnology) along with the addition of 200 µM ZnCl₂ at 16 °C. Cells were collected by centrifugation and stored at -80 °C prior to purification.

Pellets were thawed and resuspended in purification buffer [50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES; pH 7.5), 300 mM KCl, 10% glycerol (v/v), 1 mM tris(2-carboxyethyl)phosphine (TCEP)] and lysed by sonication. Lysate was clarified by centrifugation at 33,000g for 1 hour at 4 °C. The supernatant was purified using an amylose column (New England BioLabs) and protein was eluted using 10 mM maltose. Protein was digested using recombinant His-TEV protease overnight at 4 °C while dialyzing in purification buffer + 20 mM imidazole. The digest was applied to an equilibrated Ni-NTA resin (Qiagen) to remove His-MBP.
and His-TEV, which were subsequently eluted with a 0–400 mM imidazole gradient in purification buffer. The HDAC6-containing fractions were concentrated to <10 mL over a 10kDa MWCO filter unit (Millipore) and applied to a HiLoad Superdex 200pg column in size exclusion buffer [50 mM HEPES (pH 7.5), 100 mM KCl, 5% glycerol (v/v), 1 mM TCEP]. Fractions containing pure HDAC6 were identified using SDS-PAGE, pooled, and concentrated to 14–20 mg/mL. Protein was flash cooled in liquid nitrogen and stored at -80 °C prior to usage.

3.2.3 – Crystallization

All HDAC6-inhibitor complexes were crystallized in sitting drops by the vapor diffusion method at 4 °C.

For cocrystallization of the HDAC6–HPB complex, a 350 nL drop of protein solution [10 mg/mL HDAC6, 50 mM HEPES (pH 7.5), 100 mM KCl, 5% glycerol (v/v), 1 mM TCEP, 5 mM HPB, and 5% dimethyl sulfoxide (DMSO) (v/v)] was added to a 350 nL drop of precipitant solution [200 mM ammonium chloride and 20% (w/v) PEG 3,350] and equilibrated against an 80 µL reservoir of precipitant solution. Hexagonal plate crystals appeared within 2 days.

For initial cocrystallization of the HDAC6–ACY-1083 complex, a 2 µL drop of protein solution [10 mg/mL HDAC6, 100 mM HEPES (pH 7.5), 100 mM KCl, 5% glycerol (v/v), 1 mM TCEP, 5 mM ACY-1083, and 5% DMSO] was added to a 2 µL drop of precipitant solution [50 mM magnesium acetate, 100 mM sodium cacodylate (pH 6.5), and 25% (w/v) PEG 8,000] and equilibrated against a 500 µL reservoir of precipitant solution. A few rhombus-shaped crystals appeared after 1 week. These were crushed and used as a seed stock for streak-seeding cocrystallization experiment using the same conditions except a lower protein concentration was used (5 mg/mL HDAC6). This approach yielded many pyramid-shaped crystals within 1 day.
For cocrystallization of the HDAC6–Ricolinostat complex, a 350 µL drop of protein solution [10 mg/mL HDAC6, 50 mM HEPES (pH 7.5), 100 mM KCl, 5% glycerol (v/v), 1 mM TCEP, saturating Ricolinostat (ca. 1 mM), and 5% DMSO (v/v)] was added to a 350 µL drop of precipitant solution [100 mM sodium citrate (pH 5.5) and 16% (w/v) PEG 8,000] and equilibrated against an 80 µL reservoir of precipitant solution. Crystal plates appeared within 3 days.

For cocrystallization of HDAC6 with the R-stereoisomer of TSA, a 350 µL drop of protein solution [10 mg/mL HDAC6, 50 mM HEPES (pH 7.5), 100 mM KCl, 5% glycerol (v/v), 1 mM TCEP, 2 mM TSA, 5% DMSO] was added to 350 µL of precipitant solution [200 mM MgCl₂, 100 mM Bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (BisTris; pH 6.5), and 25% (w/v) PEG 3,350] and equilibrated against an 80 µL reservoir of precipitant solution. Thick crystal plates appeared within 2 days.

All crystals were soaked in a cryoprotectant solution containing mother liquor supplemented with 20% glycerol (HDAC6–TSA) or 15% (HDAC6–ACY-1083), 20% (HDAC6–HPB), or 25% (HDAC6–Ricolinostat) ethylene glycol prior to flash-cooling in liquid nitrogen. For the structural determination of HDAC6–R–TSA complexes at varied pH values, crystals were cryoprotected in precipitant solutions buffered with BisTris at pH values of 5.5, 6.0, 6.5, 7.0, and 7.5, as well as 20% ethylene glycol.

3.2.4 – Data Collection and Structure Determination

X-ray diffraction data were collected from crystals on beamline 9-2 at the Stanford Synchrotron Radiation Laboratory (SSRL), Stanford University (HDAC6–ACY-1083 and HDAC6–HPB complexes), beamline 4.2.2 at the Advanced Light Source (ALS), University of California-Berkeley (HDAC6–Ricolinostat complex), and on beamline 24-ID-C at the Advanced
Photon Source (APS), Argonne National Laboratory (HDAC6–TSA complex). Data were indexed and integrated using either XDS\textsuperscript{134} (HDAC6–TSA, HDAC6–ACY-1083, and HDAC6–Ricolinostat complexes) or iMosflm\textsuperscript{103} (HDAC6–HPB complex) and scaled using Aimless in the CCP4 program suite.\textsuperscript{104} Data collection statistics for these four structures are recorded in Table 3.1. Statistics for the HDAC6–TSA complexes at varied pH values are presented in Table 3.2.

All crystal structures were solved by molecular replacement using the atomic coordinates of unliganded HDAC6 (PDB 5EEM)\textsuperscript{48} as a search model for rotation and translation function calculations using the program Phaser.\textsuperscript{105} Atomic models were constructed using the graphics program Coot\textsuperscript{106} and crystallographic structure refinement was performed using PHENIX.\textsuperscript{107} Inhibitor molecules were added in the later stages of refinement. Occasionally, maps displayed spurious electron density peaks that could not be satisfactorily modeled by ordered solvent, in which case these were left interpreted. The overall quality of each model was assessed using MolProbity\textsuperscript{108} and PROCHECK.\textsuperscript{109} Final refinement statistics are recorded in Table 3.1 and Table 3.2.
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Values in parentheses refer to data in the highest shell. \(^*\)R_{merge} = \sum_{hkl} |I_{hkl} - \langle I_{hkl}\rangle|/\sum_{hkl} I_{hkl}, where \(\langle I_{hkl}\rangle\) is the average intensity calculated for reflection \(hkl\) from replicate measurements. \(^*\)R_{work} = \sum_{hkl} |(I_{o,hkl} - I_{c,hkl})/I_{o,hkl}|/\sum_{hkl} I_{o,hkl}, where \(I_{o,hkl}\) is the average intensity calculated for reflection \(hkl\) from replicate measurements and \(N\) is the number of reflections. \(^*\)Pearson correlation coefficient between random half-datasets. \(^*\)R_{free} = \sum |F_o | - | F_c | /\sum |F_o | for reflections contained in the working set. \([F_o]\) and \([F_c]\) are the observed and calculated structure factor amplitudes, respectively. \(^*\)Calculated with PROCHECK.\(^{109}\)
Table 3.2 | Structural statistics for HDAC6-R-TSA complexes at variant pH values

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$^{a}$Values in parentheses refer to data in the highest shell. $^{b}R_{merge} = \Sigma_{i}I_{i,kl} - (\Sigma_{i}I_{i,kl})/\Sigma_{i}I_{i,kl}$, where $(\Sigma_{i}I_{i,kl})$ is the average intensity calculated for reflection $hkl$ from replicate measurements. $^{c}R_{p.i.m.} = (\Sigma_{i}(1/(N-1))^{1/2}I_{i,kl} - (\Sigma_{i}I_{i,kl})/\Sigma_{i}I_{i,kl}$, where $(\Sigma_{i}I_{i,kl})$ is the average intensity calculated for reflection $hkl$ from replicate measurements and N is the number of reflections. $^{d}$Pearson correlation coefficient between random half-datasets. $^{e}R_{work} = \Sigma||F_o|| - |F_c||/\Sigma|F_o|| for reflections contained in the working set. $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively. $R_{free}$ is calculated using the same expression for reflections contained in the test set held aside during refinement. $^{f}$Calculated with PROCHECK. $^{109}$
3.3 | Results

3.3.1 – HDAC6–HPB Complex

The 1.82 Å-resolution structure of the HDAC6–HPB complex does not reveal any significant conformational changes relative to unliganded HDAC6 (PDB 5EEM), and the root-mean-square deviation (rmsd) between unliganded and inhibitor-bound structures is 0.14 Å for 284 Ca atoms. The hydroxamate group of HPB coordinates to the active site zinc ion only through its N–O− group (Zn2+---O distance = 1.9 Å), which binds at the coordination site that would be occupied by the substrate carbonyl in catalysis (Figure 3.2a). The catalytic zinc-bound water molecule remains in place and donates a hydrogen bond to the hydroxamate C=O group (O---O distance = 2.6 Å). This water molecule also forms hydrogen bonds with H573 and H574. Additionally, the Y745 hydroxyl group interacts with the hydroxamate NH (O---N distance = 2.6 Å) and O− (O--O distance = 2.7 Å) groups.

The phenyl linker group of HPB is sandwiched between F583 and F643 (Figure 3.2b). The peptoid capping group is observed in both the trans and cis conformations with respective occupancies of 0.66 and 0.34, corresponding to ΔG = 0.4 kcal/mol. This is within the range of 0.3–0.6 kcal/mol measured for peptoid trans-cis isomerization,135 so the enzyme does not exhibit a clear preference for one conformation or the other. The phenyl group of the cis conformer makes a van der Waals contact with L1 loop residue P464; the energetically preferred trans conformation of the peptoid moiety allows the phenyl capping group to make a quadrupole-charge interaction with the side chain of R601 in an adjacent monomer. The hydroxyl moiety of the capping group forms a water-mediated hydrogen bond with S531 (Figure 3.2a). The side chain of S531 accepts
Figure 3.2 | HDAC6–HPB complex at 1.82 Å resolution

(a) Simulated annealing omit map (green, contoured at 2.0σ) for cis (yellow) and trans (orange) conformations of HPB bound to HDAC6. Omit density is also shown for the water molecule (red sphere) bound to the Zn$^{2+}$ ion (grey sphere). Metal coordination and hydrogen bond interactions are indicated by solid and dashed black lines, respectively. (b) Molecular surface of the HDAC6 active site showing the aromatic ring of the phenylhydroxamate nestled between F583 and F643.
a hydrogen bond from the backbone NH group of the acetyl-L-lysine substrate, an interaction that is unique to the HDAC6 active site \(^4^8\).

3.3.2 – HDAC6–ACY-1083 Complex

The 1.75 Å-resolution structure of the HDAC6–ACY-1083 complex does not reveal any major structural rearrangements caused by inhibitor binding, and the rmsd is 0.16 Å for 300 Ca atoms between the inhibitor-bound and unliganded enzymes. The hydroxamate moiety adopts monodentate \(\text{Zn}^{2+}\) coordination geometry identical to that observed in the HPB complex: the hydroxamate N–O\(^-\) group coordinates to \(\text{Zn}^{2+}\) (\(\text{Zn}^{2+}---\text{O}\) distance = 1.9 Å) and interacts with Y745 (O---O distance = 2.5 Å) (Figure 3.3a). The hydroxamate C=O group accepts a hydrogen bond from the \(\text{Zn}^{2+}\)-bound water molecule (O---O distance = 2.7 Å); this water molecule also hydrogen bonds with H573 and H574. The hydroxamate NH group interacts with the side chain of Y745 (O---N distance = 2.7 Å).

The aromatic ring of the aminopyrimidine linker is sandwiched between F583 and F643 in a similar manner to that observed for the phenyl linker of HPB (Figure 3.3b). The hydroxyl side chain of S531 on the L2 loop accepts a hydrogen bond from the secondary amino group of the inhibitor (O---N distance = 3.1 Å) (Figure 3.3a). The difluorocyclohexyl capping group adopts a chair conformation, and the equatorial fluorine atom packs against the edge of the F643 side chain (F---C distance = 3.6 Å). The phenyl capping group of the inhibitor makes van der Waals interactions with the side chains of P464 and F583.

3.3.3 – HDAC6–Ricolinostat

The 1.70 Å-resolution structure of the HDAC6–Ricolinostat (ACY-1215) complex reveals that no major conformational changes are triggered by inhibitor binding, and the rmsd is
Figure 3.3 | HDAC6–ACY-1083 complex at 1.75 Å resolution

(a) Simulated annealing omit map (green, contoured at 2.5σ) for ACY-1083 (orange) and the water molecule (red sphere) bound to the Zn$^{2+}$ ion (large grey sphere) in the active site of HDAC6. Metal coordination and hydrogen bond interactions are indicated by solid and dashed black lines, respectively. (b) Molecular surface of the HDAC6 active site showing the heteroaromatic ring of the inhibitor nestled between F583 and F643. The phenyl group of the capping group lies adjacent to F583 and also P464 (not shown in this view).
0.65 Å for 336 Cα atoms between the inhibitor-bound and unliganded enzymes. The hydroxamate moiety of Ricolinostat coordinates to Zn\(^{2+}\) in bidentate fashion (Figure 3.4), forming a canonical 5-membered chelate complex with Zn\(^{2+}\)--O distances of 2.0 Å and 2.4 Å for the N--O\(^-\) and C=O groups, respectively. The side chain of Y745 donates a hydrogen bond to the hydroxamate C=O group (O--O distance = 2.7 Å), H573 donates a hydrogen bond to the hydroxamate N--O\(^-\) group (N--O distance = 2.7 Å) and H574 accepts a hydrogen bond from the hydroxamate NH group (N--N distance = 2.9 Å). Hydrogen bond interactions with the tandem histidine residues suggest that H573 is in the positively charged imidazolium state and H574 is in the neutral imidazole state. These protonation states are consistent with the assignment of the second histidine in this tandem pair as a single general base-general acid in catalysis, as recently demonstrated for HDAC8.\(^{76,77}\)

Two monomers reside in the asymmetric unit of this crystalline enzyme-inhibitor complex. In one monomer, the inhibitor capping group adopts two mutually exclusive conformations (Figure 3.4). In one conformation, the amide carbonyl of the inhibitor capping group forms a water-mediated hydrogen bond with S531 on the L1 loop. The aminopyrimidinyl ring nitrogen also forms a water-mediated hydrogen bond with D460. In the second conformation, a water-mediated hydrogen bond is formed between the amide carbonyl of the inhibitor capping group and H614 (one of the Zn\(^{2+}\) ligands). The aminopyrimidinyl ring nitrogen also forms a water-mediated hydrogen bond with the carbonyl of P711.

### 3.3.4 – HDAC6–TSA Complex

The 1.05 Å-resolution structure of HDAC6 complexed with the \(R\)-stereoisomer of TSA complex reveals significantly more detail in comparison with the previously reported 1.59 Å-resolution structure of this complex (PDB 5EEK).\(^{48}\) Although the polypeptide backbones of
Figure 3.4 | HDAC6–Ricolinostat complex at 1.70 Å resolution

Simulated annealing omit map (green, contoured at 2.5σ) for Ricolinostat bound to HDAC6 (monomer A). Two conformations for the capping group are shown with different shades of purple, the darker of which indicates the sole conformation observed in monomer B. The Zn$^{2+}$ ion is shown as a large grey sphere and water molecules are shown as smaller red spheres. Metal coordination and hydrogen bond interactions are indicated by solid and dashed black lines, respectively.
HDAC6 in the ultrahigh and high resolution structures are essentially identical (rmsd = 0.064 Å for 315 Cα atoms), the ultrahigh resolution structure reveals major and minor conformers for the hydroxamate group of TSA (Figure 3.5a). The minor conformation is not observable in crystal structures determined at lower resolution.48,49

The major conformer of TSA (70% occupancy) corresponds to that described in the 1.59 Å resolution structure, in which the canonical bidentate hydroxamate–Zn\(^{2+}\) coordination mode is observed with O---Zn\(^{2+}\) distances of 2.2 Å and 2.0 Å for the hydroxamate C=O and N–O– groups, respectively. Additionally, Y745 donates a hydrogen bond to the hydroxamate C=O, while H573 and H574 form hydrogen bonds with the N–O– group (Figure 3.5b).

The minor conformer of TSA (30% occupancy; Figure 3.5c) corresponds to the monodentate hydroxamate–Zn\(^{2+}\) coordination mode observed for sterically bulky HDAC6-selective inhibitors such as HPOB, HPB, and ACY-1083. The hydroxamate group of TSA coordinates to Zn\(^{2+}\) only through its N–O– group (Zn\(^{2+}\)---O distance = 1.8 Å), which binds at the coordination site that would be occupied by the substrate carbonyl in catalysis. Weak electron density is observed for the Zn\(^{2+}\)-bound water molecule, which donates a hydrogen bond to the hydroxamate C=O group (O---O distance = 2.8 Å). Additionally, the hydroxyl group of Y745 is within hydrogen bonding distance to the hydroxamate NH and N–O– groups with O---N and O---O distances of 2.7 Å and 2.8 Å, respectively.

Other than differences in the conformation and orientation of the hydroxamate group, no other structural features distinguish the major and minor conformers of TSA. The dimethylheptadiene linker and \(\rho\)-dimethylaminophenyl capping group bind in identical fashion in high- and low-occupancy conformations. Based on the 70:30 ratio observed for bidentate:monodentate Zn\(^{2+}\)
Figure 3.5 | HDAC6–R-TSA complex at 1.05 Å resolution

(a) Simulated annealing omit map (green, contoured at 2.5σ) of the R-stereoisomer of TSA bound to HDAC6, showing the binding of major (purple) and minor (orange) inhibitor conformations. (b) Omit map of the major TSA conformer (70% occupancy) reveals the canonical bidentate hydroxamate-\(\text{Zn}^{2+}\) coordination geometry. (c) Omit map of the minor TSA conformer (30% occupancy) reveals unusual monodentate hydroxamate-\(\text{Zn}^{2+}\) coordination geometry. The \(\text{Zn}^{2+}\) ion appears as a large grey sphere; metal coordination and hydrogen bond interactions are shown as solid and dashed black lines, respectively.
coordination modes for the hydroxamate group of TSA, the monodentate hydroxamate-Zn$^{2+}$ binding mode is only 0.5 kcal/mol higher in energy than the canonical bidentate hydroxamate-Zn$^{2+}$ binding mode. In structures determined at pH values ranging from 5.5 to 7.5, the occupancy ranges for the mono- and bidentate metal coordination geometries are 28-33% and 67-72%, respectively, indicating no change in the monodentate:bidentate ratio as a function of the environmental pH.
3.4 | Discussion

3.4.1 – Structural Aspects of HDAC6–Inhibitor Selectivity

Each component of an HDAC inhibitor – the Zn²⁺-binding group, the linker, and the capping group – contributes to the selectivity of inhibitor binding measured in biochemical assays. The key finding of the present study is that monodentate hydroxamate–Zn²⁺ coordination can be exploited by sterically bulky phenylhydroxamate inhibitors in the active site of HDAC6. The inhibitors HPB, ACY-1083, and HPOB each exhibit monodentate hydroxamate–Zn²⁺ coordination through their hydroxamate N–O groups, with their hydroxamate C=O groups hydrogen bonded to a Zn²⁺-bound water molecule (Figure 3.6). The pan-HDAC inhibitor TSA (R-stereoisomer) engages in both bidentate and monodentate hydroxamate–Zn²⁺ coordination, as enabled by its comparatively slender linker group. The bidentate:monodentate ratio of 70:30 indicates a free energy difference of only 0.5 kcal/mol, so the monodentate hydroxamate–Zn²⁺ binding mode observed exclusively for sterically bulky phenylhydroxamate inhibitors does not significantly compromise enzyme-inhibitor affinity. The energetically accessible monodentate binding mode can be exploited by inhibitors that are too bulky to bind more deeply in the HDAC6 active site as would be required for bidentate coordination. This binding mode appears to be unaffected by perturbations in pH, despite different possible protonation states of H574 in either mono- or bidentate binding, suggesting that this ratio is enforced by the structure of the inhibitor rather than variability in the protein environment.

Interestingly, the secondary amino linker group of ACY-1083 donates a hydrogen bond directly to the hydroxyl group of S531 (Figure 3.3). S531 plays a key role in HDAC6–substrate recognition by accepting a hydrogen bond from the backbone NH group of the scissile acetyl-L-lysine substrate. In class I enzymes such as HDAC8, D101 serves this role and accepts hydrogen
Figure 3.6 | Bidentate and monodentate hydroxamate–zinc binding modes

Representation of the (a) bidentate and (b) monodentate Zn$^{2+}$ binding modes observed for hydroxamate HDAC inhibitors.
bonds from both backbone NH groups flanking the scissile acetyl-L-lysine residue.\textsuperscript{73,110} While HPB lacks comparable functionality to make a direct interaction with S531, the hydroxyl group of its peptoid capping group forms a water-mediated hydrogen bond with S531 (Figure 3.2). Thus, direct or water-mediated hydrogen bonding with S531 is an interaction unique to the HDAC6 active site that confers some measure of isozyme selectivity.

The capping group of each inhibitor significantly contributes to HDAC6 affinity and selectivity. The capping group binds at the mouth of the active site cleft, and this region exhibits significant structural differences among the HDAC isozymes. In HDAC6, the L1 (H455–E465), L2 (M517–N536) and L7 (A706–Q716) loops can interact with inhibitor capping groups. The aromatic capping groups of HPB, ACY-1083, and HPOB pack primarily against the L1 loop; in addition, the capping group of Ricolinostat is sufficiently large to interact with both the L1 and L7 loops.

The L1 loops of HDAC6 and class I HDACs adopt different conformations that significantly influence inhibitor binding (Figure 3.7). The phenyl group of the 260-fold selective inhibitor ACY-1083 packs tightly against the side chains of H463 and P464 in the L1 loop. Superposition of this complex with the structures of HDAC1, HDAC2, and HDAC3 complexed with their respective activating domains shows that the L1 loops of class I HDACs adopt different conformations that constrict their active sites relative to HDAC6.\textsuperscript{44,46} This would create a steric clash with the binding conformation of ACY-1083. A similar steric clash would result with the L1 loop of HDAC2.\textsuperscript{45} Thus, ACY-1083 binding to class I HDACs appears to be disfavored due to clashes between the large capping group and the L1 loop.
Superposition of HDAC6 CD2 (pale blue) complexed with ACY-1083 (orange), HDAC1 (PDB 4BKX), HDAC2 (PDB 4LY1), and HDAC3 (PDB 4A69). All class I HDACs are shown in shades of grey. The L1 and L2 loops are shown in shades of red, orange, and yellow for HDAC1, HDAC2, and HDAC3, respectively, and shades of blue for HDAC6. Corepressor proteins complexed with HDAC1 and HDAC3, respectively MTA1 and NCOR2, are shown in green and appear to stabilize the L1 loop conformation. The inositol tetraphosphate (IP$_4$) bound at the HDAC3–NCOR2 interface is also shown. A conserved proline residue is shown on each L1 loop to highlight the steric crowding that would occur upon ACY-1083 binding to the class I HDACs. The L1 loop is set back by ca. 1 Å in HDAC6 relative to the class I HDACs. The Zn$^{2+}$ ion of HDAC6 is shown as a lavender sphere.
Ricolinostat has the largest capping group of the inhibitors shown in Table 1, and this cap binds in a cleft between the L1 and L7 loops of HDAC6. Superposition with HDAC3 shows a ∼1 Å difference in these loop conformations, resulting in a narrowed cleft that would be less ideal for Ricolinostat binding. The 12-fold HDAC6-selectivity of Ricolinostat arises solely from this capping group, since the flexible aliphatic linker and bidentate hydroxamate-\(\text{Zn}^{2+}\) binding mode are otherwise identical to those of the pan-HDAC inhibitor SAHA.

3.4.2 – HDAC6–Nexturastat A Complex

The 1.99 Å-resolution crystal structure of HDAC6 CD2 complexed with the HDAC6-selective inhibitor Nexturastat A \(136\) (NextA; Figure 3.8a) was recently reported.\(^9\) Curiously, despite its structural resemblance to HPOB and HPOB as a bulky phenylhydroxamate derivative, NextA is reported to bind with an alternative monodentate hydroxamate-\(\text{Zn}^{2+}\) coordination geometry in which the hydroxamate carbonyl oxygen coordinates to \(\text{Zn}^{2+}\) with a \(\text{Zn}^{2+}---\text{O}\) distance of 2.6 Å (Figure 3.8b). This separation is rather long for inner-sphere metal coordination. However, we inspected the electron density map generated with structure factor amplitudes and phases calculated from the final model of the enzyme-inhibitor complex deposited in the Protein Data Bank (PDB accession code 5G0I); we suggest that the map better accommodates monodentate hydroxamate-\(\text{Zn}^{2+}\) coordination by the hydroxamate N–O\(^-\) group (Figure 3.8c). This binding mode refines with a \(\text{Zn}^{2+}---\text{O}\) distance of 2.2 Å, which is more typical for an inner-sphere metal coordination interaction. Additionally, the hydroxamate C=O group accepts a hydrogen bond from the \(\text{Zn}^{2+}\)-bound water molecule, as observed for HPOB,\(^{48}\) HPB (Figure 3.2), ACY-1083 (Figure 3.3), and the minor conformer of TSA (Figure 3.5). Thus, we suggest that Figure 3.8c illustrates the preferred binding mode of NextA.
Figure 3.8 | Reinterpretation of the HDAC6–Nexturastat A complex (PDB 5G0I)

(a) Structure of Nexturastat A (NextA) shown with the same color scheme as in Figure 1 (blue = linker, orange = capping group). (b) Simulated annealing omit map contoured at 2.5σ showing NextA (orange) bound to CD2 (grey) in monomer A of the structure as reported by Matthias and colleagues (PDB 5G0I). (c) Simulated annealing omit map contoured at 2.5σ for a reinterpreted model of NextA (orange) bound to CD2 (blue). The Zn$^{2+}$ ion is shown as a grey sphere and the Zn$^{2+}$-bound water molecule is shown as a small red sphere. Metal coordination and hydrogen bond interactions are shown as solid and dashed black lines, respectively.
3.5 | Summary & Conclusions

Canonical bidentate hydroxamate-Zn\(^{2+}\) coordination was first observed for inhibitor binding to thermolysin,\(^{137}\) and exceptions are rarely observed.\(^{138}\) Here, we have outlined a new hydroxamate-Zn\(^{2+}\) binding mode exploited by bulky phenylhydroxamates in the active site of HDAC6: these inhibitors coordinate to Zn\(^{2+}\) through a monodentate hydroxamate N–O\(^-\) group while the hydroxamate C=O group accepts a hydrogen bond from Zn\(^{2+}\)-bound water. A 6-membered ring hydroxamate-Zn\(^{2+}\)-H\(_2\)O complex results, as summarized in Figure 3.6. The free energy of this novel Zn\(^{2+}\) binding mode is just 0.5 kcal/mol higher than that of the canonical bidentate hydroxamate-Zn\(^{2+}\) complex. This alternative Zn\(^{2+}\) binding mode can be exploited by bulky phenylhydroxamate inhibitors in the active site of HDAC6, even though the binding of these inhibitors would be disfavored in the more sterically constricted active sites of class I HDACs. Intriguingly, this alternative binding mode would disfavor the potential for the Zn\(^{2+}\)-dependent Lossen rearrangement that could convert the hydroxamate moiety into a mutagenic isocyanate derivative.\(^91\)

The linker groups of HDAC6-selective inhibitors can make direct or water-mediated hydrogen bonds with S531. This residue accepts a hydrogen bond from the backbone NH group of the acetyl-L-lysine substrate,\(^{48}\) and this interaction is unique to the HDAC6 active site. Thus, an inhibitor that targets this interaction will exhibit selectivity for binding to HDAC6. Finally, interactions of inhibitor capping groups at the mouth of the HDAC6 active site reveal that structural differences in the L1 loop also contribute to isozyme selectivity. Thus, specific interactions of the Zn\(^{2+}\)-binding group, linker, and capping group together contribute to selectivity for HDAC6 binding.
Chapter 4 | Influence of Capping Groups on Inhibitor Binding

Work presented here is reprinted from Porter, N.J.; Osko, J.D.; Diedrich, D.; Kurz, T.; Hooker, J.M.; Hansen, F.K.; Christianson, D.W. *J. Med. Chem.* 2018, *61*, 8054-8060. Crystal structures of HDAC6 in complex with compounds 1, 2, and Bavarostat were determined by N.J.P. The HDAC6-3 complex was determined by J.D.O. Inhibitor 3 was synthesized by D.D.

4.1 | Introduction

As mentioned in Section 3.1, the selective inhibition of histone deacetylase 6 (HDAC6) is a viable route to the treatment of a variety of clinical conditions. Selective inhibition of this enzyme, serving as the cytosolic tubulin deacetylase, can be achieved by designing hydroxamate inhibitors bearing substantial rigidity and steric bulk.

To better understand the structure–affinity and structure–selectivity relationships in HDAC6-inhibitor complexes, we determined X-ray crystal structures of catalytic domain 2 (CD2) of HDAC6 from *Danio rerio* (zebrafish) complexed with four different phenylhydroxamic acid-based inhibitors at 1.47–2.20 Å resolution. These studies focused on the inhibition of CD2 and not catalytic domain 1 (CD1) because only CD2 exhibits broad-specificity catalytic activity; moreover, it is this domain that is established to be the responsible for tubulin deacetylation. Molecular structures of the phenylhydroxamate-based inhibitors are shown in Figure 4.1. Inhibitors 1 and 2 contain large peptoid capping groups and exhibit better than 200-fold selectivity in comparison with HDAC2, whereas structurally related inhibitor 3 is essentially non-selective. Bavarostat (4) exhibits better than 16-fold selectivity in comparison with HDAC1, HDAC2, and HDAC3. Bavarostat is used as a brain-penetrant positron emission tomography (PET) probe for imaging HDAC6 in the central nervous system.
These structural studies show that peptoid inhibitors 1–3 bind to HDAC6 with primarily monodentate hydroxamate-Zn$^{2+}$ coordination geometry; however, Bavarostat (4) binds with canonical bidentate hydroxamate-Zn$^{2+}$ coordination geometry. Comparisons with available structures of HDAC6-inhibitor complexes$^{48,49,142}$ suggest that the hybridization of the linker benzylic nitrogen and the steric bulk of the capping group influence the denticity of hydroxamate-Zn$^{2+}$ coordination. Interactions of the capping group in a pocket defined by the L1 loop further contribute to selectivity for binding to HDAC6.
Figure 4.1 | Phenylhydroxamate-based HDAC6 inhibitors

Phenylhydroxamate-based HDAC6 inhibitors and the corresponding selectivity data over the class I enzyme HDAC1. Literature values obtained from: aRef. 140; bRef. 141.
4.2 | Materials & Methods

4.2.1 - Reagents.

Chemicals used in buffer and crystallization conditions were obtained from Fisher, Milli-pore Sigma, or Hampton Research and used without further purification. Inhibitors 1 and 2 were synthesized as reported.\textsuperscript{140} Bavarostat was synthesized as described.\textsuperscript{141} Inhibitor 3 was synthesized through a similar approach as used for the synthesis of inhibitors 1 and 2, except that benzyl isocyanide was used as the isocyanide component and 4-(dimethylamino)benzoic acid was used as the carboxylic acid component in the Ugi four-component reaction. Product purity was confirmed to be greater than 95% based on RP-HPLC analysis. The compound characterization data of compound 3 are summarized below.

\textit{N-(2-(Benzylamino)-2-oxoethyl)-4-(dimethylamino)-N-(4-(hydroxycarbamoyl)benzyl)benzamide} (3). Colorless solid; mp: 193 °C; purity: 98.2 %. \textsuperscript{1}H NMR (300 MHz, DMSO-d\textsubscript{6}): \textit{δ} = 11.20 (s, 1H), 9.03 (s, 1H), 8.60-8.32 (m, 1H), 7.83-7.66 (m, 2H), 7.46-7.14 (m, 9H), 6.75-6.55 (m, 2H), 4.67 (s, 2H), 4.30 (d, \textit{J} = 5.8 Hz, 2 H), 3.90 (s, 2H), 2.93 (s, 6H) ppm. \textsuperscript{13}C NMR (75 MHz, DMSO-d\textsubscript{6}): \textit{δ} = 171.7, 168.15, 164.0, 151.2, 140.8, 139.2, 131.7, 128.6, 128.3, 127.3, 127.15, 127.0, 126.82, 126.86, 121.9, 110.95, 42.1, 39.7 ppm. Anal. Calcd. for C\textsubscript{26}H\textsubscript{29}N\textsubscript{4}O\textsubscript{4}: 461.2183 [M+H]\textsuperscript{+}, found: 461.2182.

4.2.2 - Inhibitory activities.

The \textit{in vitro} inhibitory activities (IC\textsubscript{50} values) of compounds 1, 2, and 4 (Bavarostat) against HDAC isozymes have been previously reported.\textsuperscript{140,141} The \textit{in vitro} inhibitory activities of compound 3 against HDAC6 and HDAC1 were measured using a previously described protocol.\textsuperscript{143} OptiPlate-96 black microplates (Perkin Elmer) were used with an assay volume of 50 µL. A total
of 5 µL 3 or control, diluted in assay buffer [50 mM Tris-HCl (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 0.1 mg/mL BSA], were incubated with 35 µL of the fluorogenic substrate ZMAL (Z-Lys(Ac)-AMC) (21.43 µM in assay buffer) and 10 µL of human recombinant HDAC1 (BPS Bioscience, Catalog #50051) or HDAC6 (BPS Bioscience, Catalog #50006) at 37 °C. After an incubation time of 90 min, 50 µL of 0.4 mg/mL trypsin in trypsin buffer [50 mM Tris-HCl (pH 8.0), 100 mM NaCl] were added, followed by further incubation at 37 °C for 30 min. Fluorescence was measured with an excitation wavelength of 355 nm and an emission wavelength of 460 nm using a Fluoroskan Ascent microplate reader (Thermo Scientific). Compound 3 was evaluated in duplicate in two independent experiments.

4.2.3 - Crystallization.

Catalytic domain 2 of HDAC6 from Danio rerio (henceforth simply “HDAC6”) was recombinantly expressed in the E. coli BL21 (DE3) strain using the His₆-MBP-TEV-HDAC6-pET28a(+) vector and purified as previously described. All HDAC6-inhibitor complexes were crystallized in sitting drops by the vapor diffusion method at 4°C.

For cocryrstallization of the HDAC6–1 complex, a 5 µL drop of protein solution [5 mg/mL HDAC6, 50 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) (pH 7.5), 100 mM KCl, 5% glycerol (v/v), 1 mM tris(2-carboxyethyl)phosphine (TCEP), saturated 1 (approximately 0.5 mM), and 5% DMSO (v/v)] was added to 5 µL of precipitant solution [400 mM NaF and 15% polyethylene glycol (PEG) 3,350 (w/v)] and equilibrated against 500 µL of precipitant solution. Crystals appeared within 2 days.

For cocryrstallization of the HDAC6–2 complex, a 350 nL drop of protein solution [10 mg/mL HDAC6, 50 mM HEPES (pH 7.5), 100 mM KCl, 5% glycerol (v/v), 1 mM TCEP, saturated 2 (approximately 1.0 mM), and 5% DMSO (v/v)] was added to 350 nL of precipitant
solution [2% tacsimate (pH 6.0; w/v), 0.1 M BisTris (pH 6.5), and 20% PEG 3,350 (w/v)] and equilibrated against 80 µL of precipitant solution. Crystals appeared within 2 days.

For cocrystallization of the HDAC6–3 complex, a 350 nL drop of protein solution [10 mg/mL HDAC6, 50 mM HEPES (pH 7.5), 100 mM KCl, 5% glycerol (v/v), 1 mM TCEP, saturated 3 (approximately 0.5 mM), and 5% DMSO (v/v)] was added to 350 nL of precipitant solution [0.2 M ammonium tartrate dibasic pH 7.0 and 20% PEG 3,350 (w/v)] and equilibrated against 80 µL of precipitant solution. Crystals appeared within 2 days.

For cocrystallization of the HDAC6–4 (Bavarostat) complex, a 350 nL drop of protein solution [10 mg/mL HDAC6, 50 mM HEPES (pH 7.5), 100 mM KCl, 5% glycerol (v/v), 1 mM TCEP, saturated Bavarostat (approximately 0.5 mM), and 5% DMSO (v/v)] was added to 350 nL of precipitant solution [200 mM L-proline, 100 mM HEPES (pH 7.5), and 24% PEG 1,500 (w/v)] and equilibrated against 80 µL of precipitant solution. Crystals appeared within 2 days.

All crystals were soaked in a cryoprotectant solution containing mother liquor supplemented with 20% ethylene glycol prior to flash-cooling in liquid nitrogen.

4.2.4 - Data collection and structure determination.

X-ray diffraction data for HDAC6 complexes with 1 and 2 were collected on NE-CAT beamline 24-ID-E at the Advanced Photon Source, Argonne National Lab. Diffraction data were collected from crystals of the HDAC6–3 and HDAC6–4 complexes on beamline 9-2 at the Stanford Synchrotron Radiation Laboratory (SSRL), Stanford University. Data were indexed and integrated using iMosflm\textsuperscript{103} and data scaling was carried out using Aimless in the CCP4 program suite.\textsuperscript{104}

The atomic coordinates of unliganded HDAC6 (PDB 5EEM)\textsuperscript{48} were used as a search model to phase each crystal structure by molecular replacement using the program Phaser.\textsuperscript{105}
Atomic models were built and manipulated using the graphics program Coot\textsuperscript{106} and crystallographic refinement was executed using Phenix.\textsuperscript{107} Inhibitor molecules were built into well-defined electron density in later rounds of refinement. The quality of each model was assessed using MolProbity\textsuperscript{108} and PROCHECK.\textsuperscript{109} Data collection and refinement statistics are recorded in Table 4.1.
Table 4.1 | Structural statistics for HDAC6 complexes with bulky inhibitors

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<td>78.6, 95.5, 98.3</td>
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<td>90.0, 90.0, 120.0</td>
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<td>0.129 (0.723)</td>
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<td>6DVM</td>
<td>6DVN</td>
<td>6DVO</td>
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Values in parentheses refer to data in the highest shell. ¹Rmerge = \( \sum_{hk} \left| I_{hk} - \langle I_{hk} \rangle \right| / \sum_h \sum_k I_{hk} \), where \( \langle I_{hk} \rangle \) is the average intensity calculated for reflection \( hkl \) from replicate measurements. ²Rprep = \( \left( \sum_h \sum_k \sum_{i=1}^{N} \left| I_{hk} - \langle I_{hk} \rangle \right| / \sum_h \sum_k \sum_{i=1}^{N} I_{hk} \right) \), where \( \langle I_{hk} \rangle \) is the average intensity calculated for reflection \( hkl \) from replicate measurements and \( N \) is the number of reflections. ³Pearson correlation coefficient between random half-datasets. ⁴Rwork = \( \sum_{hk} \left| F_o - F_c \right| / \sum_{hk} |F_o| \) for reflections contained in the working set. \( |F_o| \) and \( |F_c| \) are the observed and calculated structure factor amplitudes, respectively. Rfree is calculated using the same expression for reflections contained in the test set held aside during refinement. ⁵Calculated with PROCHECK, ¹⁰⁹

85
4.3 | Results and Discussion

The 2.10 Å resolution structure of the HDAC6–1 complex reveals monodentate hydroxamate–Zn$^{2+}$ coordination in both monomers in the asymmetric unit (Figure 4.2a). Selected interatomic distances are recorded in Table 4.2. The phenyl linker is nestled in the aromatic crevice formed by F583 and F643 with average inter-ring separations of 3.5 Å each. The peptoid carbonyl is oriented away from “gatekeeper” residue S531 in the L2 loop and accepts a hydrogen bond from the guanidinium group of R601 in an adjacent monomer. This places the dimethylphenyl substituent in van der Waals contact with the side chains of L1 loop residues H463 and P464, which define a small pocket designated the “L1 loop pocket”. The cyclohexylamide carbonyl forms hydrogen bonds with two water molecules, one of which interacts with the backbone carbonyl of A641 while the other forms a hydrogen bond with another water molecule that interacts with Zn$^{2+}$ ligand H614. The cyclohexyl moiety is oriented away from the enzyme surface, packing against the side chain of R736 in the adjacent monomer in the crystal lattice.

The crystal structure of the HDAC6–2 complex determined at 1.47 Å resolution reveals a monodentate hydroxamate–Zn$^{2+}$ binding mode in monomers A, C, and D in the asymmetric unit generally similar to that of compound 1; selected interatomic distances are recorded in Table 1. In monomer B, electron density is consistent with a mixture of monodentate and bidentate hydroxamate–Zn$^{2+}$ binding modes with refined occupancies of 0.63 and 0.37, respectively (Figure 4.2b). The lower occupancy bidentate conformation exhibits Zn$^{2+}$--O distances of 2.5 Å for the hydroxamate C=O and 2.2 Å for the hydroxamate N–O$^-$ group.

Inter-ring separations between the phenyl linker of 2 and the side chains of F583 and F643 are 3.3 Å and 3.9 Å, respectively, and the capping group adopts an essentially identical conformation for both metal-binding modes. The peptoid carbonyl is oriented away from the enzyme.
Figure 4.2 | Polder omit maps of bulky hydroxamates complexed with HDAC6

Polder omit maps (green mesh) contoured at 3.0σ for compounds (a) 1 (orange), (b) 2 (orange/purple), (c) 3 (yellow), and (d) Bavarostat (blue) complexed with HDAC6 (white). The catalytic Zn^{2+} ion appears as a gray sphere; metal coordination and hydrogen bond interactions are shown as solid and dashed black lines, respectively. Atoms from a symmetry-related molecule in the crystal lattice are shown with dark-gray carbon atoms. In b, the monodentate hydroxamate conformation of 2 is shown in orange and the bidentate conformation is shown in purple. The binding of 1 (a) and 3 (c) illustrates exclusive monodentate hydroxamate-Zn^{2+} coordination; the binding of 4 (Bavarostat) (d) illustrates exclusive bidentate hydroxamate-Zn^{2+} coordination.
Table 4.2 | Average interatomic distances in monodentate inhibitor complexes (Å)

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<th>Interatomic Measurement</th>
<th>Inhibitor</th>
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<td>N–O⁻---Zn²⁺</td>
<td>2.1</td>
</tr>
<tr>
<td>H₂O---Zn²⁺</td>
<td>2.1</td>
</tr>
<tr>
<td>C=O---OH₂</td>
<td>2.8</td>
</tr>
<tr>
<td>O⁻---O (Y745)</td>
<td>2.5</td>
</tr>
<tr>
<td>H₂O---N (H573)</td>
<td>2.6</td>
</tr>
<tr>
<td>H₂O---N (H574)</td>
<td>2.9</td>
</tr>
</tbody>
</table>
surface toward bulk solvent. The dimethylaniline group resides in the L1 loop pocket, and its nitrogen atom makes a van der Waals contact with P464. Interestingly, crystal packing places the dimethylaniline moieties of separate inhibitor molecules adjacent to each other, such that they form a staggered π-stacking interaction with a ring separation distance of 3.9 Å. The tolylamide carbonyl interacts with Zn$^{2+}$ ligand H614 through two hydrogen bonded water molecules, as observed for compound 1. The tolyl moiety is accordingly oriented away from the enzyme surface and makes a van der Waals contact with the dimethylaniline methyl group of an inhibitor bound to an adjacent monomer in the crystal lattice.

The crystal structure of the HDAC6–3 complex determined at 2.20 Å resolution largely resembles that of the HDAC6–2 complex. All four monomers in the asymmetric unit uniformly adopt monodentate hydroxamate-Zn$^{2+}$ coordination geometry (Figure 4.2c, Table 4.2). The phenyl linker resides in the aromatic crevice and is 3.3 Å and 4.0 Å away from the phenyl groups of F583 and F643, respectively. The peptoid carbonyl is oriented away from the protein surface and out toward bulk solvent. As in the HDAC6–2 complex, the dimethylaniline group resides in the L1 loop pocket. The dimethylaniline groups in adjacent monomers form staggered π-stacking interactions with a ring separation of 3.8 Å. The benzylamide carbonyl engages in the same solvent-mediated interaction with Zn$^{2+}$ ligand H614 as observed for the binding of compound 2. The benzyl moiety of compound 3 also makes a van der Waals contact with the dimethylaniline group of another inhibitor molecule bound to an adjacent monomer in the crystal lattice.

The crystal structure of the HDAC6–4 (Bavarostat) complex determined at 1.98 Å resolution, containing only one monomer in the asymmetric unit, clearly reveals canonical bidentate hydroxamate-Zn$^{2+}$ coordination geometry with Zn$^{2+}$---O separations of 2.2 and 2.0 Å, respectively, for the hydroxamate C=O and N−O$^-$ groups (Figure 4.2d). The catalytic histidine residues,
H573 and H574, form hydrogen bonds with the hydroxamate N–O– and NH groups (N_{573}--O separation = 2.5 Å and N_{574}--N separation = 2.8 Å). The Y745 phenol oxygen hydrogen bonds with the hydroxamate carbonyl (O--O separation = 2.4 Å). The 2-fluorophenyl linker is situated in the aromatic crevice such that the fluorine atom is 3.3 Å from the side-chain methylene group of S531, 3.1 Å from the Cα atom of G582, 3.6 Å from the side chain of F583, and 3.1 Å from the side chain of F643. The benzylic tertiary amine is pyramidalized such that the lone pair on the nitrogen is oriented away from the side chain of gatekeeper residue S531, which revises a prior prediction. Instead, this nitrogen is oriented toward bulk solvent. The adamantyl capping group is nestled in the L1 loop pocket.

4.3.1 *The HDAC6 L1 loop pocket.*

As the structures of HDAC6 complexed with compounds 1, 2, and 3 are compared, common features are evident for the binding of inhibitor capping groups. First, capping groups are similarly oriented, in that the smaller peptoid substituent (dimethylphenyl for 1, dimethylaniline for 2 and 3) sits in the L1 loop pocket while the larger peptoid substituent is oriented away from the protein surface. Curiously, the crystal structure of isolated compound 1 (CSD ID: GADYOC) exhibits a *cis* peptoid conformation, whereas it exhibits a *trans*-peptoid conformation in its complex with HDAC6 (Figure 4.3a). The lower free energy difference between peptoid conformers presumably enables *cis*-trans isomerism to optimize the fit of the enzyme-inhibitor complex. The association of the smaller peptoid substituent with the L1 loop pocket thus appears to direct the peptoid conformation (Figure 4.3b). Notably, the adamantyl group of Bavaroostat is similarly positioned in the L1 loop pocket (Figure 4.3c), as are cap substituents of other HDAC6-selective inhibitors. Taken together, these data indicate that the L1 loop pocket of HDAC6 provides a binding site for hydrophobic capping groups. Key residues defining this
Figure 4.3 | Binding of compound 1 to HDAC6 and packing in the L1 loop pocket

(a) Stereo superposition of crystal structures of free (light blue) and HDAC6-complexed (orange) conformations of compound 1. The phenyl linkers of these models have been aligned, highlighting conformational differences in the capping groups. (b) Binding of compound 1 to HDAC6 (light blue) with the dimethylphenyl group of the inhibitor cap packing against the L1 loop (H455–E465; yellow). (c) Binding of 4 (Bavarostat, blue) to HDAC6 viewed from a similar orientation to that shown in (b).
pocket (H463, P464, F583, and L712) are strictly conserved between human HDAC6, the actual drug target, and zebrafish HDAC6, the ortholog used for X-ray crystallographic studies.

Notably, the L1 loop of HDAC6 is relatively rigid. Accordingly, the rigid, pre-formed nature of the L1 loop pocket presumably contributes to the selectivity of inhibitor binding to HDAC6 by minimizing the entropic cost of inhibitor binding site organization. Indeed, the binding of selective inhibitors to HDAC6 is generally characterized by entropy gain.145

These results further support the hypothesis that interactions with the L1 loop are important for HDAC6–inhibitor selectivity. In the class I HDACs 1, 2, and 3, the L1 loop is shifted by approximately 1 Å relative to HDAC6, constricting the substrate binding pocket, and the L1 loop is buttressed when the enzyme is activated through the binding of corepressor and inositol tetraphosphate.44–46 This conformational difference would perturb the binding of a sterically bulky inhibitor capping group, thus rendering the inhibitor ineffective. We suggest that this effect accounts for the selectivity of Bavarostat for inhibition of HDAC6 relative to the class I HDACs 1, 2, and 3 (Figure 4.1).141

Surprisingly, inhibitor 3 binds with comparable affinity to HDAC6 compared with inhibitor 2, but 3 is much less selective than 2 with respect to inhibition of class I HDACs. We attribute this to the additional flexibility conferred by the benzylic substituent of 3 compared with the more rigid tolyl substituent of 2. The additional bulk and flexibility of 3 presumably enables binding to the more constricted active sites of class I HDACs, as exemplified for HDAC3 in Figure 4.4.

4.3.2 - Capping group influence on hydroxamate–Zn\(^{2+}\) denticity.

Compared to other HDACs, the substrate binding groove of HDAC6 is wider (Figure 4.4). As a result, sterically bulky phenylhydroxamates can readily access the catalytic Zn\(^{2+}\) ion of HDAC6 but they cannot interact as easily with the catalytic Zn\(^{2+}\) ion of class I HDAC isoymes.
Active site surfaces of (a) HDAC6 in its complex with inhibitor 1, and (b) HDAC3 (PDB 4A69) with inhibitor 1 modeled in the active site based on structural alignment with the HDAC6–1 complex. Zn\(^{2+}\) ions appear as grey spheres and metal coordination interactions are represented by solid yellow lines. The active site of HDAC6 is wider and more readily accommodates phenylhydroxamate inhibitors with bulky *para*-substituted substituents.
However, the molecular features that distinguish monodentate versus bidentate hydroxamate-Zn$^{2+}$ coordination in the HDAC6 active site are less clear.

Insight regarding this phenomenon can be acquired from studying the 22 unique hydroxamate based crystal structures of HDAC6–inhibitor complexes determined to date, including the 4 structures reported here. Of these, 11 display canonical bidentate binding, 9 display monodentate binding, and 2 exhibit fractional occupancy of each conformation in one crystallographic monomer. Bidentate metal coordination is generally observed for inhibitors that possess either flexible aliphatic linkers or aromatic linkers lacking a capping group. Addition of bulky and/or rigid capping groups to a phenylhydroxamate inhibitor generally leads to monodentate hydroxamate-Zn$^{2+}$ coordination, but the steric bulk must be located close to the phenylhydroxamate moiety. Specifically, the capping group must branch at the second atom away from the phenyl ring, and both substituents at the branch must be bulky. While Bavarostat (4) contains a bulky adamantyl cap as one substituent at the amino branch of the capping group, the second substituent is only a methyl group, which thus enables the phenylhydroxamate to more closely approach the catalytic Zn$^{2+}$ ion to achieve bidentate coordination geometry. Another feature that may enable binding flexibility for Bavarostat is the $sp^3$-hybridized nitrogen atom of the tertiary amino group itself. In contrast with the planar $sp^2$-hybridized peptoid nitrogen atoms of inhibitors 1–3, the tertiary amino nitrogen of Bavarostat can rapidly invert between two pyramidal stereoisomers as needed to optimize enzyme-inhibitor interactions.

Compounds 1–3 contain relatively rigid peptoid moieties with two bulky substituents branching at the second atom away from the para-substituted phenylhydroxamate. Apart from one of four monomers in the HDAC6–2 complex, these inhibitors bind with monodentate hydroxamate-Zn$^{2+}$ coordination geometry. Taking into account multiple independent copies of the
enzyme-inhibitor complex with 2 in the asymmetric unit, the overall monodentate:bidentate ratio is 3.6:0.4. This suggests that, for compound 2, monodentate coordination is ~1.3 kcal/mol more stable as observed in the crystal structure. In comparison, the mixture of monodentate and bidentate conformers in the 1.05 Å-resolution structure of the HDAC6–R-TSA complex suggested that bidentate coordination was 0.5 kcal/mol more stable. Therefore, it appears that the steric bulk and rigidity of inhibitor capping groups modulate the equilibrium between bidentate and monodentate hydroxamate–Zn$^{2+}$ coordination and thereby direct the metal ion coordination mode.
4.4 | Conclusions

The high-resolution crystal structures of HDAC6 complexes with bulky peptoid inhibitors 1–3 as well as Bavarostat (4) highlight the importance of the L1 loop pocket in accommodating large hydrophobic groups. This pocket is largely defined by H463, P464, F583, and L712, and these residues are conserved between human HDAC6, the actual human drug target, and zebrafish HDAC6, the ortholog used for X-ray crystal structure determinations. Each inhibitor studied is a phenylhydroxamate containing a capping group that branches at the second atom away from the aromatic ring. Analysis of HDAC6-inhibitor complexes suggests that if both branching substituents are sterically bulky, then the inhibitor will bind with monodentate hydroxamate-Zn$^{2+}$ coordination geometry; if only one substituent is bulky, or if there is no substituent at all (i.e., a simple phenylhydroxamate$^{26}$), then the inhibitor will bind with bidentate hydroxamate-Zn$^{2+}$ coordination geometry. Future studies will allow us to further clarify structure-affinity and selectivity relationships for inhibitor binding to HDAC6.
Chapter 5 | Entropy Contributes to HDAC6-Selective Inhibition

Work presented here is reprinted from Porter, N.J.; Wagner, F.F.; Christianson, D.W. *Biochemistry* 2018, 57, 3916-3924.

5.1 | Introduction

As discussed in chapters 3 and 4, typical HDAC inhibitors consist of a zinc-binding group such as a hydroxamate, a capping group capable of interacting with residues in the active site cleft, and a linker group connecting the two. Appreciable selectivity for HDAC6 is observed for inhibitors containing aromatic linker groups and bulky capping groups such as Tubastatin A,\textsuperscript{129} N-hydroxy-4-(2-[(2-hydroxyethyl)(phenyl)amino]-2-oxoethyl)benzamide (HPOB),\textsuperscript{130} N-hydroxyl-4-[(N(2-hydroxyethyl)-2-phenylacetamido)methyl]-benzamide)] (HPB),\textsuperscript{131} and Nexturastat.\textsuperscript{136} The previously discussed crystal structures of HDAC6 complexed with some of these inhibitors have revealed that these sterically bulky inhibitors exploit an unusual monodentate hydroxamate-Zn\textsuperscript{2+} coordination mode that is nearly isoenergetic with the more commonly observed bidentate hydroxamate-Zn\textsuperscript{2+} coordination mode.\textsuperscript{48,49,142} This binding mode results from steric constrictions in the HDAC6 active site by the L1, L2, and L6 loops that prevent phenylhydroxamates with bulky capping groups from making a closer interaction with the catalytic Zn\textsuperscript{2+} ion. However, simple “capless” inhibitors retain nanomolar potency and 10-fold or greater selectivity for HDAC6 relative to class I HDACs based on IC\textsubscript{50} assays (Figure 5.1).\textsuperscript{148} Therefore, the bulky capping group is not the sole determinant of selectivity. What, then, determines the isozyme selectivity of these low-molecular weight, capless inhibitors?
To address this question, we determined X-ray crystal structures of the high-activity CD2 domain from *Danio rerio* HDAC6 (henceforth simply “HDAC6”) complexed with the capless inhibitors phenylhydroxamate (1), cyclohexenylhydroxamate (2), cyclohexylhydroxamate (3), and cyclopentenylhydroxamate (4) (Figure 5.1). These compounds have been previously profiled as HDAC6 inhibitors with sub-micromolar potencies and selectivities of up to 36-fold based on IC₅₀ measurements made by Wagner and colleagues. To better understand selective HDAC inhibition by these compounds, we also performed thermodynamic measurements of enzyme-inhibitor complexation using isothermal titration calorimetry (ITC). These studies together reveal that a favorable entropy of binding contributes to the inhibitory selectivity towards the class IIb enzyme HDAC6 over the class I enzyme HDAC8.
Figure 5.1 | Capless HDAC6-selective inhibitors

Structure and selectivity of capless cyclohydroxamate inhibitors that are selective for HDAC6 over HDAC8 in IC$_{50}$ assays. Literature values obtained from Ref. 148.

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<td>380 ± 60</td>
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<tr>
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<td>1900 ± 400</td>
<td>430 ± 80</td>
<td>4000 ± 1000</td>
<td>1100 ± 100</td>
</tr>
</tbody>
</table>

Selectivity: 16  36  10  37
5.2 | Materials and Methods

5.2.1 – Reagents

In general, chemicals used in buffers and crystallization conditions were purchased from Fisher, Millipore Sigma, or Hampton Research and used without further purification. Compounds 1–4 were synthesized as described.\textsuperscript{148}

5.2.2 – Protein Preparation

Catalytic domain 2 from \textit{Danio rerio} HDAC6 (herein designated simply “HDAC6”) was recombinantly expressed using His\textsubscript{6}-MBP-TEV-HDAC-pET28a(+) vectors and purified as previously described with minor modifications.\textsuperscript{142} Briefly, HDAC6 was expressed using \textit{E. coli} BL21 (DE3) (Stratagene) grown in 2x YT medium under the selection of 50 mg/mL kanamycin. Expression was induced with 250 µM isopropyl β-L-1-thiogalactopyranoside (IPTG; Gold Biotechnology) along with the addition of 500 µM ZnCl\textsubscript{2} at 18˚C. Cells were collected by centrifugation and stored at -80 ˚C prior to purification.

Pellets were thawed and resuspended in purification buffer [50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES; pH 7.5), 300 mM KCl, 10% glycerol (v/v), 1 mM tris(2-carboxyethyl)phosphine (TCEP)] and lysed by sonication. Lysate was clarified by centrifugation at 38,000g for 1 hour at 4 ˚C. The supernatant was applied to amylose resin (New England BioLabs). His\textsubscript{6}-MBP-TEV-HDAC6 fusion protein was either eluted using 10 mM maltose or digested on-column with 2 mg/mL recombinant His-TEV protease. Eluted fusion protein was digested using recombinant His-TEV protease overnight at 4 ˚C while dialyzing in purification buffer. The digest was applied to an equilibrated Ni-NTA agarose column (Qiagen) to remove His-MBP and His-TEV, which were subsequently eluted using a 0–500 mM imidazole gradient in purification buffer. The HDAC6-containing fractions were concentrated to <10 mL over a
10,000 Da molecular weight cut-off filter unit (Millipore) and applied to a HiLoad Superdex 200pg column in size exclusion buffer [50 mM HEPES (pH 7.5), 100 mM KCl, 5% glycerol (v/v), 1 mM TCEP]. Fractions containing pure HDAC6 were identified using SDS-PAGE, pooled, and concentrated to 14–20 mg/mL. Protein was flash-frozen in liquid nitrogen and stored at -80 °C prior to usage.

HDAC8 was expressed and purified as previously described, with minor modifications. Briefly, a 500 mL culture [Lysogeny Broth (LB) at 100 µg/mL ampicillin] was grown overnight at 37 °C with shaking at 250 rpm. Aliquots of this culture (30 mL) were used to inoculate 12 × 1 L of M9 minimal medium supplemented with 100 µg/mL ampicillin. Cells were grown until the OD_{600} ≈ 1.0, at which point the temperature was reduced to 18 °C. After cooling for 30 min, protein expression was induced with 100 µM ZnCl₂ and 100 µM IPTG. Protein was expressed overnight and pellets were harvested via centrifugation and stored at -80 °C until they were purified. The purification was carried out as previously described using a Co²⁺-TALON® column followed by size exclusion chromatography. All protein was concentrated to 10-20 mg/mL, flash-frozen in liquid nitrogen, and stored at -80 °C until use.

5.2.3 – Crystallization

All HDAC6–inhibitor complexes were crystallized in sitting drops by the vapor diffusion method at 4 °C.

For cocrystallization of the HDAC6–1 and HDAC6–2 complexes, a 350 nL drop of protein solution [5 mg/mL HDAC6, 50 mM HEPES (pH 7.5), 100 mM KCl, 5% glycerol (v/v), 1 mM TCEP, 5 mM 1 or 2, and 5% dimethyl sulfoxide (DMSO) (v/v)] was added to 350 nL of precipitant solution [200 mM ammonium chloride and 20% PEG 3,350] and equilibrated against 80 µL of precipitant solution. Rhomboid plate crystals appeared within 2 days.
For cocrystallization of the HDAC6–3 complex, a 2 µL drop of protein solution [2.5 mg/mL HDAC6, 50 mM HEPES (pH 7.5), 100 mM KCl, 5% glycerol (v/v), 1 mM TCEP, 5 mM 3, and 5% dimethyl sulfoxide (DMSO) (v/v)] was added to a 2 µL drop of precipitant solution [40 mM sodium citrate/60 mM Bis-Tris propane (pH 6.4) and 25% PEG 3,350] and was streak-seeded with a seed stock of crushed HDAC6–3 crystals previously generated under the same conditions but with 5 mg/mL enzyme in the protein solution. This was equilibrated against 80 µL of precipitant solution. Rhomboid plate crystals appeared within 2 days.

For cocrystallization of the HDAC6–4 complex, a 350 nL drop of protein solution [5 mg/mL HDAC6, 50 mM HEPES (pH 7.5), 100 mM KCl, 5% glycerol (v/v), 1 mM TCEP, 5 mM 4, and 5% dimethyl sulfoxide (DMSO) (v/v)] was added to 350 nL of precipitant solution [200 mM ammonium tartrate dibasic and 20% PEG 3,350] and equilibrated against 80 µL of precipitant solution. Rhomboid plate crystals appeared within 2 days.

All crystals were soaked in a cryoprotectant solution containing mother liquor supplemented with 20% ethylene glycol prior to flash-cooling in liquid nitrogen.

5.2.4 – Data collection and structure determination

X-ray diffraction data were collected from crystals on beamline 9-2 at the Stanford Synchrotron Radiation Laboratory (SSRL), Stanford University for the HDAC6 complexes with inhibitors 1, 2, and 3. Diffraction data for the HDAC6–4 complex were collected on beamline 17-ID-2 (FMX) at the National Synchrotron Light Source II (NSLS2), Brookhaven National Lab. Data were indexed and integrated using iMosflm and scaled using Aimless in the CCP4 program suite. Data collection statistics are recorded in Table 1. Each crystal structure was solved by molecular replacement using the atomic coordinates of unliganded HDAC6 (PDB 5EEM) as a search model for rotation and translation function calculations using the program Phaser.
Atomic models were constructed and modified using the graphics program Coot\textsuperscript{106} and crystallographic structure refinement was performed using Phenix.\textsuperscript{107} Inhibitor molecules were added when clearly resolved electron density was observed for each. Occasionally, maps displayed spurious electron density peaks that could not be satisfactorily modeled by ordered solvent, ligand, or cryoprotectant, in which case these peaks were left uninterpreted. The quality of each model was assessed using MolProbity\textsuperscript{108} and PROCHECK.\textsuperscript{109} Final refinement statistics are recorded in Table 5.1.

5.2.5 – Isothermal Titration Calorimetry

Thermograms were measured for inhibitor binding to HDAC6 and HDAC8 using a MicroCal iTC 200 isothermal titration calorimeter (GE Healthcare). For each compound, 300 µM inhibitor was titrated against 30 µM enzyme in size exclusion buffer with 0.3% DMSO for compounds 1–4. Thirty-eight 1-µL injections were made over 80 min. Integration, curve fitting, and figure generation were performed using Origin (OriginLab, Northampton, MA).
Table 5.1 | Structural statistics for HDAC6 complexes with capless cyclohydroxamates

<table>
<thead>
<tr>
<th>Complex</th>
<th>1 (phenylhydroxamate)</th>
<th>2 (cyclohexenylhydroxamate)</th>
<th>3 (cyclohexylhydroxamate)</th>
<th>4 (cyclopentenylhydroxamate)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Unit Cell</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Space group</td>
<td>P2₁2₁2₁</td>
<td>P2₁2₁2₁</td>
<td>P2₁2₁2₁</td>
<td>P2₁2₁2₁</td>
</tr>
<tr>
<td>(a, b, c) (Å)</td>
<td>74.8, 91.9, 96.4</td>
<td>74.7, 91.8, 96.5</td>
<td>74.7, 91.8, 96.5</td>
<td>74.8, 92.0, 96.6</td>
</tr>
<tr>
<td><strong>Data Collection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>0.97946</td>
<td>0.97946</td>
<td>0.97946</td>
<td>0.97933</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>48.2 – 1.62</td>
<td>59.08 – 1.24</td>
<td>96.60 – 2.03</td>
<td>96.64 – 1.70</td>
</tr>
<tr>
<td>Total / unique no. of reflections</td>
<td>528011 / 84908</td>
<td>1156727 / 188513</td>
<td>271026 / 43532</td>
<td>507763 / 74006</td>
</tr>
<tr>
<td>(R_{	ext{merge}})</td>
<td>0.189 (0.816)</td>
<td>0.101 (0.593)</td>
<td>0.228 (0.538)</td>
<td>0.224 (1.558)</td>
</tr>
<tr>
<td>(R_{	ext{pim}})</td>
<td>0.082 (0.346)</td>
<td>0.044 (0.257)</td>
<td>0.099 (0.229)</td>
<td>0.91 (0.670)</td>
</tr>
<tr>
<td>(CC_{1/2})</td>
<td>0.969 (0.682)</td>
<td>0.997 (0.871)</td>
<td>0.988 (0.850)</td>
<td>0.991 (0.605)</td>
</tr>
<tr>
<td>(I/\langle I \rangle)</td>
<td>6.7 (2.2)</td>
<td>10.0 (3.0)</td>
<td>6.3 (3.6)</td>
<td>8.6 (3.4)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>6.2</td>
<td>6.1 (6.2)</td>
<td>6.2 (6.4)</td>
<td>6.9 (6.3)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.5 (99.7)</td>
<td>99.7 (99.9)</td>
<td>99.8 (100)</td>
<td>100 (100)</td>
</tr>
</tbody>
</table>

**Refinement**

| No. of reflections used in refinement / test set | 84,783 / 8,378 | 188,358 / 18,683 | 43,445 / 4,284 | 73,824 / 7,278 |
| \(R_{	ext{work}}\) | 0.170 (0.262) | 0.127 (0.177) | 0.176 (0.221) | 0.169 (0.220) |
| \(R_{	ext{free}}\) | 0.219 (0.338) | 0.153 (0.219) | 0.223 (0.266) | 0.194 (0.256) |
| No. of nonhydrogen atoms: |
| protein | 5721 | 5838 | 5563 | 5689 |
| ligand | 74 | 125 | 26 | 60 |
| solvent | 764 | 935 | 648 | 654 |
| Average B-factors (Å²) |
| protein | 11 | 10 | 10 | 10 |
| ligand | 17 | 20 | 7 | 19 |
| solvent | 23 | 24 | 17 | 23 |
| Root-mean-square deviation from ideal geometry |
| bonds (Å) | 0.006 | 0.008 | 0.003 | 0.003 |
| angles (°) | 0.8 | 1.0 | 0.6 | 0.7 |
| Ramachandran plot (%) |
| favored | 97.75 | 97.61 | 97.60 | 97.61 |
| allowed | 2.25 | 2.39 | 2.40 | 2.39 |
| PDB accession code | 6CSR | 6CSP | 6CSQ | 6CSS |

\(^a\)Values in parentheses refer to data in the highest shell. \(^b\)\(R_{	ext{merge}} = \Sigma_{\|hkl\|} |I_{\|hkl\|} - \langle I_{\|hkl\|}\rangle|/\Sigma_{\|hkl\|} |I_{\|hkl\|}|\), where \(\langle I_{\|hkl\|}\rangle\) is the average intensity calculated for reflection \(hkl\) from replicate measurements. \(^c\)\(R_{	ext{pim}} = (\Sigma_{\|hkl\|}(1/(N-1))^{1/2}\Sigma_{\|hkl\|} |I_{\|hkl\|} - \langle I_{\|hkl\|}\rangle|/\Sigma_{\|hkl\|} |I_{\|hkl\|}|\), where \(\langle I_{\|hkl\|}\rangle\) is the average intensity calculated for reflection \(hkl\) from replicate measurements and \(N\) is the number of reflections. \(^d\)Pearson correlation coefficient between random half-datasets. \(^e\)\(R_{	ext{work}} = \Sigma |F_{o} - |F_{c}|\)/\Sigma |F_{o}| for reflections contained in the working set. \(|F_{o}|\) and \(|F_{c}|\) are the observed and calculated structure factor amplitudes, respectively. \(^f\)\(R_{	ext{free}}\) is calculated using the same expression for reflections contained in the test set held aside during refinement. Calculated with PROCHECK.\(^{109}\)
5.3 | Results and Discussion

5.3.1 – X-ray crystallography

Crystal structures were solved and refined with $R_{\text{work}}$ and $R_{\text{free}}$ values ranging 0.127–0.176 and 0.153–0.223, respectively (Table 5.1). For each HDAC6–inhibitor complex, there are no major conformational changes relative to unliganded HDAC6 (PDB 5EEM; root-mean-square deviation (rmsd) = 0.16–0.17 Å for 290–305 Cα atoms).

In the crystal structure of the HDAC6–phenylhydroxamate 1 complex determined at 1.62 Å resolution, the inhibitor hydroxamate group adopts canonical bidentate Zn$^{2+}$ coordination, forming a 5-membered ring chelate complex with Zn$^{2+}$—O distances of 2.0 Å and 2.4 Å for the N–O$^-$ and C=O groups, respectively (Figure 5.2a). This coordination geometry was first observed in the binding of hydroxamate inhibitors to the Zn$^{2+}$ hydrolase thermolysin. In the HDAC6 active site, the hydroxamate oxyanion, NH group, and C=O group also form hydrogen bonds with H573, H574, and Y745, respectively (intermolecular distances are recorded in Table 5.2). The phenyl ring of the inhibitor is firmly nestled in an aromatic crevice formed by the side chains of F563 and F643; the phenyl ring of the inhibitor is offset so that the partial positive charges of its ring hydrogen atoms interact with the partial negative charges of the ring π electrons of F583 and F643. The dihedral angle between the inhibitor phenyl ring and the hydroxamate moiety is twisted 34˚ away from planarity.

The crystal structure of the HDAC6–cyclohexenylhydroxamate 2 complex determined at 1.24 Å resolution similarly reveals bidentate hydroxamate–Zn$^{2+}$ coordination, with Zn$^{2+}$—O distances of 2.0 Å and 2.2 Å for the ionized hydroxyl and carbonyl groups, respectively (Figure 5.2b). Hydrogen bond interactions with the Zn$^{2+}$-bound hydroxamate are similar to those observed for
Figure 5.2 | Polder omit maps for capless cyclohydroxamates bound to HDAC6

Polder omit maps (green) contoured at 4.0σ for inhibitors (a) 1, (b) 2, (c) 3, and (d) 4 bound to HDAC6. Atoms are color-coded as follows: C = orange (inhibitor) or light blue (HDAC6), N = blue, O = red, Zn\(^{2+}\) = gray sphere. Metal coordination and hydrogen bond interactions are indicated by solid and dashed black lines, respectively.
Table 4.2 | Average interatomic distances in HDAC6-cyclohydroxamate complexes (Å)

<table>
<thead>
<tr>
<th>Interatomic Measurement</th>
<th>Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>C=O---Zn²⁺</td>
<td>2.4</td>
</tr>
<tr>
<td>N−O⁻---Zn²⁺</td>
<td>2.0</td>
</tr>
<tr>
<td>N---N(H574)</td>
<td>2.7</td>
</tr>
<tr>
<td>O⁻---N(H573)</td>
<td>2.8</td>
</tr>
<tr>
<td>C=O---O(Y745)</td>
<td>2.4</td>
</tr>
</tbody>
</table>
phenylhydroxamate 1 (Table 5.2). The cyclohexenyl ring of the inhibitor is bound such that the olefin moiety is firmly nestled in the F583-F643 aromatic crevice. The dihedral angle between the olefin moiety and the hydroxamate is twisted 18° away from planarity.

The crystal structure of the HDAC6–cyclohexylhydroxamate 3 complex determined at 2.03 Å resolution reveals nearly perfect bidentate hydroxamate-Zn$^{2+}$ coordination, with Zn$^{2+}$---O distances of 2.1 Å and 2.2 Å for the ionized hydroxyl and carbonyl groups, respectively (Figure 5.2c). Hydrogen bond interactions with the Zn$^{2+}$-bound hydroxamate are similar to those observed for phenylhydroxamate (Table 5.2). The cyclohexyl group adopts a chair conformation and packs between the side chains of F583 and F643. The dihedral angle between the adjacent C-C bond in the cyclohexyl ring and the hydroxamate is twisted 8° away from planarity.

Finally, the crystal structure of the HDAC6–cyclopentenylhydroxamate 4 complex determined at 1.70 Å resolution reveals canonical bidentate hydroxamate-Zn$^{2+}$ coordination geometry with Zn$^{2+}$---O distances of 2.0 Å and 2.4 Å for the ionized hydroxyl and carbonyl groups, respectively (Figure 5.2d). Hydrogen bond interactions with the Zn$^{2+}$-bound hydroxamate are similar to those observed for phenylhydroxamate (Table 5.2). The cyclopentenyl ring of the inhibitor is bound such that the olefin moiety is also firmly nestled in the F583-F643 aromatic crevice. The cyclopentenyl-hydroxamate dihedral angle is twisted 26° away from planarity.

Three interesting structural features emerge from analysis of the binding modes of capless inhibitors 1–4. First, each inhibitor binds with its hydroxamate group coordinated to Zn$^{2+}$ with canonical bidentate geometry. Notably, many HDAC6-selective inhibitors such as HPB (Figure 5.3) contain a core phenylhydroxamate moiety as represented by inhibitor 1. Depending on the capping group attached to the phenylhydroxamate, bidentate or monodentate hydroxamate coordination to the catalytic Zn$^{2+}$ ion will result, with the monodentate coordination mode being only
Figure 5.3 | Superposition of HPB linker with phenylhydroxamate (1)

Chemical structure of HPB and stereoview of the superposition of the experimentally determined structure of the HDAC6–HPB complex (PDB 5WGK; blue) with a model of HPB in the same conformation with its phenylhydroxamate moiety superimposed with that observed in the HDAC6–1 complex. Phenylhydroxamate 1 is shown in yellow while the modeled HPB cap is shown in orange.
0.5 kcal/mol less stable than the bidentate coordination mode (Figure 5.1). For example, HPB exhibits monodentate Zn$^{2+}$ coordination; least-squares superposition of the crystal structures of the HDAC6–HPB (PDB ID: 5WGK) and HDAC6–1 complexes (rmsd = 0.12 over 283 Cα atoms) reveals slight differences in the orientation of the aromatic ring (Figure 5.3). Specifically, the aromatic ring of the capless phenylhydroxamate 1 is tilted by approximately 10°, which allows a closer approach to the catalytic Zn$^{2+}$ ion, which in turn enables bidentate hydroxamate-Zn$^{2+}$ coordination.

Superposition of the HPB phenylhydroxamate moiety with compound 1 demonstrates that no particular interaction of the capping group appears to govern the hydroxamate-Zn$^{2+}$ binding mode. If HPB were tilted so as to superimpose its phenylhydroxamate moiety with that of capless phenylhydroxamate 1 in the HDAC6–1 complex (Figure 5.3), the bulky capping group would not clash with any active site residues. Thus, it is not clear how the geometry of hydroxamate-Zn$^{2+}$ coordination might be influenced by interactions of the capping group of HPB.

Second, while compounds 1, 2, and 4 contain C=C bonds potentially capable of conjugation with the π system of the hydroxamate moiety, their respective C=C–C=O dihedral angles are distorted 36°, 11°, and 46° from planarity. These values lie within the broad distribution observed for similar compounds retrieved from the Cambridge Structural Database (Figure 5.4), so there does not appear to be a strong energetic driving force to maintain planarity of the extended π system.

Finally, the binding orientations of cyclohexenylhydroxamate 2 and cyclopentenylhydroxamate 4 unambiguously place the C=C bond in the F583–F643 aromatic crevice, as also observed for the aromatic ring of phenylhydroxamate 1. Therefore, this aromatic crevice preferentially accommodates a planar olefin moiety contained in a 6- or 5-membered ring, including a 6-memb-
Deviations from planarity observed in conjugated aromatic hydroxamic acids. A total of 145 independent examples were found in 87 structures retrieved from the Cambridge Structural Database. The search query is shown as an inset structure and the angle measured is highlighted in yellow.
ered aromatic ring. The chair-conformation 6-membered ring of cyclohexylhydroxamate 3 is not as readily accommodated based on previously measured\textsuperscript{148} IC\textsubscript{50} values (Figure 5.5). Of the linker groups represented in the current study, it appears that the cyclohexenyl group of inhibitor 2 would be most ideal for further derivatization to enhance HDAC6 selectivity.

5.3.2 – Isothermal Titration Calorimetry (ITC)

To gain additional insight on the molecular basis of affinity and selectivity for inhibitor binding to HDAC6, we studied the thermodynamics of enzyme-inhibitor association using ITC. We also studied inhibitor binding to the representative class I isozyme HDAC8 using ITC to evaluate the extent of HDAC6 selectivity against class I HDACs. Titrations of inhibitors 1–4 against HDAC6 and HDAC8 reveal that the ITC-derived dissociation constants (K\textsubscript{d}) generally correlate (within a factor of 4) with previously measured\textsuperscript{148} IC\textsubscript{50} values for each enzyme-inhibitor complex (Figure 5.5).

The selectivity of the best capless inhibitors for HDAC6 compared with HDAC8 appears to be rooted in a substantial and favorable entropic gain for HDAC6 association. Specifically, the –T\Delta S term is negative and favorable for the binding of inhibitors 1–4 to HDAC6, whereas –T\Delta S is positive and unfavorable for the binding of these inhibitors to HDAC8 with the exception of compound 3 (Figure 5.5, Figure 5.6). In other words, the binding of cycloalkenyl hydroxamates 1, 2, and 4 to HDAC6 is accompanied by a substantial entropy gain, whereas the binding of these cycloalkenyl hydroxamates to HDAC8 is accompanied by an entropic loss. The outlier is the cycloalkane hydroxamate 3, which exhibits a modest and favorable entropy of binding to HDAC8; however, this compound also exhibits the weakest affinities for HDAC8 and HDAC6 and the poorest selectivity for HDAC6 (Figure 5.5). Thus, planar cycloalkenyl hydroxamates yield optimal
Figure 5.5 | Selectivity of hydroxamate inhibitors for HDAC6 versus HDAC8

IC<sub>50</sub> values compared with K<sub>d</sub> values derived from ITC measurements for "capless" HDAC6-selective inhibitors 1–4 and "capped" inhibitors SAHA, Ricolinostat, and HPB. IC<sub>50</sub> values are abstracted from ref. 46 (1–4), ref. 151 (SAHA), ref. 132 (Ricolinostat), and ref. 131 (HPB). K<sub>d</sub> data derive from isothermal titration calorimetry.
Figure 5.6 | Thermodynamic values for the binding of hydroxamate inhibitors

Thermodynamic values derived from ITC measurements for the binding of inhibitors 1–4, SAHA, Ricolinostat (R’stat), and HPB to HDAC6 CD2 (top) and HDAC8 (bottom).
affinities and HDAC6/HDAC8 selectivities of 16-fold up to 313-fold, and binding entropy appears to drive this selectivity.

Presuming identical conformations for the binding of compounds 1–4 in the active sites of other HDAC isozymes, we speculate that differences in the entropy of inhibitor binding to HDAC6 and HDAC8 could be rooted in differences in conformational entropy and/or desolva- tion entropy involving the F583-F643 aromatic crevice of HDAC6, which is conserved in HDAC8 as F152 and F208. Crystal structures of the class I isozymes HDAC1, HDAC2, and HDAC3,44–46 and the class IIa isozymes HDAC4 and HDAC7,47,50 reveal that this aromatic crevice is similarly conserved. However, structural comparisons of all isozymes reveal that relative to HDAC6, the aromatic crevice is approximately 1 Å wider in class I and class IIa HDACs. Moreover, there is evidence of conformational flexibility for one wall of this crevice: upon the binding of a bulky phenylhydroxamate inhibitor to HDAC8, the side chain of F152 rotates to adopt a conformation similar to that of the corresponding phenylalanine residue, F679, in the aromatic crevice of HDAC7.50,149 It is conceivable that inhibitor binding rigidifies the conformation of the aromatic crevice in HDAC8, thereby accounting for a conformational entropy loss. This possibility may also extend to other class I and class IIa HDACs. If the narrower aromatic crevice of HDAC6 is more rigid, then inhibitor binding would not incur a conformational entropic cost and the favorable entropy gain from active site desolvation could dominate the binding thermodynamics.

To assess the influence of a capping group on the thermodynamics of HDAC6/HDAC8 selectivity, we additionally studied the binding of the phenylhydroxamate derivative HPB,131 the aliphatic hydroxamate inhibitor Ricolinostat,132 and the aliphatic hydroxamate inhibitor subero-
lanilide hydroxamic acid (SAHA). The ITC-derived inhibitor dissociation constants (K_d) generally correlated with IC_{50} values for HDAC8 (within a factor of 4), but those for HDAC6 do not (7–32-fold variations are observed). Regardless, each of these inhibitors exhibits selectivity for binding to HDAC6 compared with HDAC8 (Figure 5.5), and selectivity is characterized by a favorable entropic gain upon binding to HDAC6 and an unfavorable entropic loss upon binding to HDAC8 (Figure 5.5, Figure 5.6). The results for HPB binding indicate that the HDAC6 selectivity inherent in the binding of capless cycloalkenyl hydroxamate inhibitors is generally maintained, but no inhibitor studied here surpasses the 313-fold thermodynamic selectivity based on K_d values measured for cyclohexenylhydroxamate 2 (Figure 5.5). Even so, it is interesting that favorable binding entropy to HDAC6 is not exclusive to inhibitors bearing aromatic or cycloalkenyl linker groups, but also includes inhibitors bearing aliphatic linker groups such as Ricolinostat and SAHA.
5.4 | Conclusions

The binding thermodynamics of compounds 1–4 to HDAC6 versus HDAC8 reflect that entropy is a key contributor to HDAC6-inhibitor binding selectivity; moreover, compounds bearing a single double bond in the ring adjacent to the hydroxamate moiety are more selective for HDAC6 than their aromatic or saturated capless counterparts. In particular, cycloalkenyl hydroxamate 2 exhibits 313-fold selective tighter binding to HDAC6 compared with HDAC8 (Figure 5.5).

The olefin moiety of 2 is preferentially accommodated in the F583–F643 aromatic crevice of HDAC6. It is not clear, however, whether the entropic favorability contributing to HDAC6 selectivity is associated with binding in this crevice, since these aromatic residues are also conserved in HDAC8 as well as other class I HDAC isoymes. However, the active site of HDAC8 is slightly larger than that of HDAC6, with an 8 Å separation between F152 and F208 in the aromatic crevice, compared with the 7 Å separation between F583 and F643 in the aromatic crevice of HDAC6. Additionally, F152 in HDAC8 exhibits conformational flexibility in complex with a bulky phenylhydroxamate inhibitor, which expands the F152–F208 separation to 8.5 Å. Possibly, the conformational flexibility of the aromatic crevice in HDAC8 contributes to the generally unfavorable entropy of inhibitor binding evident in Figure 5.6. In HDAC6, there is no evidence for conformational flexibility in the aromatic crevice, so the favorable entropy of inhibitor binding may be linked solely to desolvation.

Notably, the F583–F643 aromatic crevice of HDAC6 preferentially accommodates planar olefins, and this crevice does not accommodate the chair-conformation cyclohexyl hydroxamate 3 as readily. This inhibitor exhibits the lowest inhibitory potency, affinity, and HDAC6 selectivity
among the compounds studied (Figure 5.5). Thus, hydroxamate inhibitor designs using core cyclohexenyl hydroxamate 2 would represent an ideal starting point for the design of HDAC6 inhibitors with high affinity and selectivity.
Chapter 6 | HDAC6-Selective Inhibition by Mercaptoacetamides


6.1 | Introduction

Including those discussed chapters 3, 4, and 5, nearly all of the inhibitors that have been structurally characterized in complex with HDAC6 bear hydroxamate Zn$^{2+}$-binding groups. However, the genotoxicity associated with the hydroxamate group, such as that of the classic HDAC inhibitor suberoylanilide hydroxamic acid (SAHA,\textsuperscript{150} formulated as the cancer chemotherapy drug Vorinostat\textsuperscript{29}), argues against the use of a hydroxamate-containing inhibitor as a long-term therapy for diseases other than cancer.\textsuperscript{91,152} The chemical basis of genotoxicity derives from the Lossen rearrangement (Figure 6.1), which yields a reactive isocyanate intermediate capable of covalently modifying cellular components.\textsuperscript{91} This undesirable chemistry has motivated the search for HDAC inhibitors with alternative zinc-binding groups.

Notably, inhibitors bearing mercaptoacetamide zinc-binding groups are not genotoxic and exhibit superior neuroprotective properties.\textsuperscript{153} Moreover, certain mercaptoacetamide inhibitors exhibit nanomolar affinity and better than $10^3$-fold selectivity against HDAC6.\textsuperscript{154,155} Preceding the work presented here, the structural basis for HDAC6 affinity and selectivity not been defined.

To structurally characterize the underpinnings of this selectivity, we determined the 1.85 Å resolution structure of complex between mercaptoacetamide inhibitor N-[(5-(5,6-dichloro-1H)indol-1-yl)pentyl]-2-mercaptoacetamide (MCA; Figure 6.1)\textsuperscript{155} and catalytic domain 2 from \textit{Danio rerio} (zebrafish). MCA exhibits 240-fold selectivity for the inhibition of HDAC6 over
HDAC8. The work detailed here describes how mercaptoacetamides can be used to exploit a subtle chemical difference between HDAC isozymes which contributes to their function as HDAC6-selective inhibitors.
Figure 6.1 | Mercaptoacetamide selectivity and the hydroxamate Lossen rearrangement

(a) Structures and selectivity data for SAHA and MCA alongside (b) the mechanism for the Lossen rearrangement as potentially catalyzed by the Zn\textsuperscript{2+} ion in the HDAC active site.
6.2 | Materials and Methods

6.2.1 - Reagents.

In general, chemicals used in buffers and crystallization were purchased from Fisher, Millipore Sigma, or Hampton Research and used without further purification. The inhibitor MCA was synthesized according to published procedures.155

6.2.2 - Expression and purification of HDAC6 and HDAC8 for assay.

Full-length wild-type human HDAC6 (residues 1-1215; UniProtKB - Q9UBN7) and HDAC8 (residues 1-377; UniProtKB - Q9BY41) were prepared essentially as described previously.23,156 Briefly, expression plasmids encoding individual HDACs flanked by the N-terminal Strep-FLAG-HALO tag were constructed using the Gateway-based cloning protocol. Recombinant proteins were heterologously expressed in HEK-293/T17 following the polyethylenimine-mediated transient transfection. Three days after transfection cells were harvested, lysed in a lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 10 mM KCl, 2 mM MgCl2, 10% glycerol, and 0.2% Igepal-630] supplemented with benzonase (2 U/ml; Merck, Darmstadt, Germany) and a cocktail of protease inhibitors (Roche, Basel, Switzerland). The cell lysate was cleared by centrifugation (40,000xg for 30 mins at 4°C) and the supernatant was loaded onto a Strep-Tactin column (IBA, Gottingen, Germany) previously equilibrated in the lysis buffer. Following extensive washing with the lysis buffer, fusion proteins were eluted with the elution buffer supplemented with 3 mM desthiobiotin. The N-terminal tag was removed by the addition of the 1:20 (w/w) TEV protease overnight at 4°C and proteins further purified by size exclusion chromatography using the Superdex 16/600 HR200 column with 30 mM HEPES (pH 7.4), 140 mM NaCl, 10 mM
KCl, 3% glycerol, and 0.25 mM TCEP, as a mobile phase. Proteins were concentrated to approximately 1 mg/mL and aliquots were flash frozen in liquid nitrogen and stored at -80°C until further use. Final HDAC preparations have >95% purity as determined by SDS-PAGE.

6.2.3 - Inhibitory potencies against HDAC6 and HDAC8.

The inhibition constants were determined using a fluorogenic assay essentially as described previously. Briefly, the inhibitor MCA was preincubated with optimized concentrations of individual HDACs at 37°C for 15 min in the assay buffer [50 mM HEPES (pH 7.4), 140 mM NaCl, 10 mM KCl, 1 mg/mL bovine serum albumin, and 1 mM TCEP] in the total volume of 20 µL in 384-well plates. The deacetylation reaction was started by addition of 10 µL of acetyl-Gly-Ala-[acetyl-Lys]-AMC (HDAC6) or Boc-[trifluoroacetyl-Lys]-AMC (HDAC8) to a final concentration of 10 µM. Following a 30-min incubation at 37°C, the reaction was stopped by the addition of trypsin solution (10 µL; 2 mg/mL) and after 15-min incubation at 37°C, a fluorescence signal of released aminomethylcoumarin was quantified using a CLARIOstar fluorimeter with excitation/emission wavelengths set at 365/440 nm, respectively. Data were fit using the GraphPad Prism software and IC$_{50}$ values were calculated by non-linear regression analysis. The inhibitor and enzyme-free controls were defined as 100% and 0% HDAC activity, respectively.

6.2.4 - Expression and purification of HDAC6 for crystallization.

HDAC6 catalytic domain 2 from Danio rerio (henceforth simply "HDAC6") was recombinantly expressed using the MBP-TEV-z6CD2-pET28a(+) vector and purified as previously described with minor modification. Briefly, HDAC6 was expressed using Escherichia coli strain BL21 (DE3) (Stratagene) in 2x YT medium under the selection of 50 mg/L kanamycin. Expression was induced by 100 µM isopropyl β-D-1-thiogalactopyranoside (IPTG; Gold Biotechnology)
and 200 µM ZnCl₂ at 16 °C. Cells were collected by centrifugation and stored at -80 °C prior to purification.

Pellets were thawed and resuspended in lysis buffer [50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES; pH 7.5), 300 mM KCl, 10% glycerol (v/v), 1 mM tris(2-carboxyethyl)phosphine (TCEP)] and lysed by sonication. Lysate was clarified by centrifugation at 33,000 g for 1 hour at 4 °C. The supernatant was applied to an amylose column (New England BioLabs) and protein was eluted using 10 mM maltose. Protein was digested using recombinant His-TEV protease overnight at 4 °C while dialyzing in purification buffer supplemented with 20 mM imidazole. The digest was applied to equilibrated Ni-NTA resin (Qiagen) to remove His-MBP and His-TEV, which were subsequently eluted with a 0–400 mM imidazole gradient in purification buffer. The HDAC6-containing fractions were concentrated to <10 mL over a 10-kD molecular weight cut-off filter unit (Millipore) and applied to a HiLoad Superdex 200pg column in size exclusion buffer [50 mM HEPES (pH 7.5), 100 mM KCl, 5% glycerol (v/v), 1 mM TCEP]. Fractions containing pure HDAC6 were identified using SDS-PAGE, pooled, and concentrated to 14–20 mg/mL. Protein was flash cooled in liquid nitrogen and stored at -80 °C prior to usage.

For cocrystallization of the HDAC6–MCA complex, a 3.5 µL drop of protein solution [10 mg/mL HDAC6, 50 mM HEPES (pH 7.5), 100 mM KCl, 5% glycerol (v/v), 1 mM TCEP, saturated MCA (~1 mM), and 5% dimethyl sulfoxide (DMSO) (v/v)] was added to a 4.0 µL drop of precipitant solution [0.04 M citrate/0.06 M BisTris propane (pH 6.4) and 20% (w/v) PEG 3,350] and equilibrated against a 500 µL reservoir of precipitant solution. Rhomboid plate-like crystals appeared within 2 days. Crystals were soaked in a cryoprotectant solution consisting of mother liquor supplemented with 20% ethylene glycol prior to flash-cooling in liquid nitrogen.
6.2.5 - *Crystallographic data collection and structure determination.*

X-ray diffraction data were collected from crystals on beamline 24-ID-E at the Advanced Photon Source (APS), Argonne National Laboratory (HDAC6–TSA complex). Data were indexed and integrated using iMosflm\textsuperscript{103} and scaled using Aimless in the CCP4 program suite.\textsuperscript{104} Data collection statistics are recorded in Table S1.

The crystal structure was solved by molecular replacement using the atomic coordinates of unliganded HDAC6 (PDB 5EEM)\textsuperscript{48} as a search model for rotation and translation function calculations using the program Phaser.\textsuperscript{105} Atomic models were constructed using the graphics program Coot\textsuperscript{106} and crystallographic structure refinement was performed using Phenix.\textsuperscript{107} Inhibitor molecules in monomers A and B in the asymmetric unit were added in the later stages of refinement. Occasionally, maps displayed spurious electron density peaks that could not be satisfactorily modeled by ordered solvent, in which case were left uninterpreted. The overall quality of each model was assessed using MolProbity\textsuperscript{108} and PROCHECK.\textsuperscript{109} Final refinement statistics are recorded in Table 6.1.
Table 6.1 | Structural statistics for the HDAC6–MCA complex

<table>
<thead>
<tr>
<th>HDAC6–MCA</th>
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</thead>
<tbody>
<tr>
<td><strong>Unit Cell</strong></td>
</tr>
<tr>
<td>Space group</td>
</tr>
<tr>
<td>a, b, c (Å)</td>
</tr>
<tr>
<td>(\alpha, \beta, \gamma (°))</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Data Collection</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength (Å)</td>
</tr>
<tr>
<td>Resolution (Å)</td>
</tr>
<tr>
<td>Total / unique no. of reflections</td>
</tr>
<tr>
<td>(R_{\text{merge}}^{ab})</td>
</tr>
<tr>
<td>(R_{\text{p.i.m.}}^{ac})</td>
</tr>
<tr>
<td>(CC_{1/2}^{ad})</td>
</tr>
<tr>
<td>I/(\sigma(I)^a)</td>
</tr>
<tr>
<td>Redundancy(^a)</td>
</tr>
<tr>
<td>Completeness (%)(^a)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Refinement</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of reflections used in refinement / test set</td>
</tr>
<tr>
<td>(R_{\text{work}}^{ae})</td>
</tr>
<tr>
<td>(R_{\text{free}}^{ae})</td>
</tr>
<tr>
<td>No. of nonhydrogen atoms:</td>
</tr>
<tr>
<td>protein</td>
</tr>
<tr>
<td>ligand</td>
</tr>
<tr>
<td>solvent</td>
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<table>
<thead>
<tr>
<th>Average (B)-factors (Å(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>protein</td>
</tr>
<tr>
<td>ligand</td>
</tr>
<tr>
<td>solvent</td>
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<table>
<thead>
<tr>
<th>Root-mean–square deviation from ideal geometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>bonds (Å)</td>
</tr>
<tr>
<td>angles (°)</td>
</tr>
<tr>
<td>Ramachandran plot (%)(^f)</td>
</tr>
<tr>
<td>favored</td>
</tr>
<tr>
<td>allowed</td>
</tr>
</tbody>
</table>

| PDB accession code | 6MR5 |

\(^a\)Values in parentheses refer to data in the highest shell. \(^b\)\(R_{\text{merge}} = \Sigma_{hkl} \Sigma_{i} |I_{i,hkl} - \langle I_{hkl}\rangle| / \Sigma_{hkl} \Sigma_{i} |I_{i,hkl}|\), where \(\langle I_{hkl}\rangle\) is the average intensity calculated for reflection \(hkl\) from replicate measurements. \(^c\)\(R_{\text{p.i.m.}} = (\Sigma_{hkl}(1/(N-1))^{1/2}\Sigma_{i} |I_{i,hkl} - \langle I_{hkl}\rangle|) / \Sigma_{hkl} \Sigma_{i} |I_{i,hkl}|\), where \(\langle I_{hkl}\rangle\) is the average intensity calculated for reflection \(hkl\) from replicate measurements and \(N\) is the number of reflections. \(^d\)Pearson correlation coefficient between random half-datasets. \(^e\)\(R_{\text{work}} = \Sigma |F_{o} - |F_{c}|| / \Sigma |F_{o}|\) for reflections contained in the working set. \(|F_{o}|\) and \(|F_{c}|\) are the observed and calculated structure factor amplitudes, respectively. \(R_{\text{free}}\) is calculated using the same expression for reflections contained in the test set held aside during refinement. \(^f\)Calculated with PROCHECK.\(^{109}\)
6.3 | Results and Discussion

The 1.85 Å resolution structure of the HDAC6-MCA complex was determined to characterize isozyme-selective HDAC inhibition by an inhibitor bearing a non-hydroxamate warhead. Crystallographic refinement converged smoothly to \( R_{\text{work}}/R_{\text{free}} = 0.190/0.226 \); data collection and refinement statistics are recorded in Table 6.1. The overall structure of the polypeptide chain in the HDAC6-MCA complex is essentially identical to that in the unliganded enzyme (root-mean-square deviation = 0.2 Å over 292 Cα atoms), indicating that no major structural rearrangements are required for the binding of MCA.

An electron density map showing the bound inhibitor is presented in Figure 6.2. The thiol group of MCA is presumably ionized to the negatively charged thiolate and coordinates to the active site Zn\(^{2+}\) ion such that the overall metal coordination geometry is slightly distorted tetrahedral, with ligand–Zn\(^{2+}\)–ligand angles ranging 94°–127° across both monomers in the asymmetric unit of the crystal; metal coordination geometry deviates from ideal tetrahedral geometry by an average deviation of 10 ± 5° for ligand–Zn\(^{2+}\)–ligand bond angles. For monomers A and B, the Zn\(^{2+}\)--S separations are 2.3 Å, the C–S–Zn\(^{2+}\) angles are 114° and 120°, and the C–C–S–Zn\(^{2+}\) dihedral angles are 14° and 2°, respectively. Apart from the cisoid C–C–S–Zn\(^{2+}\) dihedral angle, the Zn\(^{2+}\) coordination geometry is ideal as outlined for thiolate-metal coordination interactions in refined protein structures.\(^{157}\) In comparison, the Zn\(^{2+}\)-bound thiolate group of the cyclic depsipeptide Largazole thiol exhibits similar coordination geometry except for a more favorable C–C–S–Zn\(^{2+}\) dihedral angle of 92° in its complex with HDAC8.\(^{92}\)

The zinc-bound thiolate group of MCA accepts a hydrogen bond from the side chain of H573 (N\(\varepsilon_{573}\)--S separation = 3.1 Å), which in turn donates a hydrogen bond to D610 (N\(\delta_{573}\)---
Figure 6.2 | Binding of MCA to HDAC6 catalytic domain 2

(a) Polder omit map (4.0σ; green mesh) for MCA (orange) bound to HDAC6 (blue). Hydrogen bond and metal-coordination interactions are shown as dashed and solid lines, respectively. The active site zinc ion is represented as a gray sphere. (b) Schematic representation of active site interactions for MCA bound to HDAC6.
O$_{610}$ separation = 2.8 Å), thus confirming that the side chain of H573 is in the positively charged imidazolium state. H573 is the first histidine in a tandem pair found in all HDAC active sites. Similar interactions are observed in the crystal structure of *Schistosoma mansoni* HDAC8 (SmHDAC8; 42% identity with human HDAC8) complexed with a mercaptoacetamide analog of SAHA, in which the Zn$^{2+}$-bound thiolate group accepts a hydrogen bond from H141 (average N$_{e141}$---S separation for monomers A–D = 3.3 Å) (Figure 3).

In the HDAC6-MCA complex, the mercaptoacetamide carbonyl oxygen accepts a hydrogen bond from the phenolic hydroxyl group of Y745 (O$_{745}$---O separation = 2.4 Å). This interaction mimics the role of Y745 in polarizing the scissile carbonyl group of acetyl-L-lysine. However, the carbonyl oxygen of MCA is 2.0 Å away from the Zn$^{2+}$ coordination site ordinarily required for substrate binding, such that the Zn$^{2+}$---O separation is 3.4 Å. Since both metal coordination and hydrogen bond interactions are required to activate the scissile amide group for hydrolysis, the amide group of the mercaptoacetamide is rendered chemically inert through its binding geometry in the HDAC6 active site.

The mercaptoacetamide NH group donates a hydrogen bond to N$_{e}$ of H574 (N$_{e574}$---N separation = 3.2 Å and 3.0 Å in monomers A and B, respectively), which requires that the side chain of H574 is in the neutral imidazole form. This is the second histidine in the tandem pair; intriguingly, the corresponding interaction with the second histidine, H142, in the mercaptoacetamide complex with SmHDAC8 is too long for hydrogen bonding (N$_{e}$---N separations range 3.5–4.0 Å in monomers A–D). Structures of mercaptoacetamide inhibitors complexes with HDAC6 and SmHDAC8 are compared in Figure 6.3. The lack of a hydrogen bond with H142 in the SmHDAC8-mercaptoacetamide complex may be due to the side chain of H142 being protonated as the positively charged imidazolium cation.
Figure 6.3 | Comparison of mercaptoacetamide binding to HDAC6 and HDAC8

Structures of (a) *S. mansoni* HDAC8 (dark red; PDB ID 4CQF) complexed with a mercaptoacetamide analog of SAHA (yellow) and (b) the HDAC6-MCA complex (colors match Figure 2) showing interactions in the active site of each enzyme. Hydrogen bond and metal-coordination interactions are shown as dashed and solid lines respectively.
The role of the second histidine in the tandem pair as a hydrogen bond acceptor, requiring a neutral imidazole side chain, is similarly required for the binding of hydroxamate inhibitors with bidentate Zn$^{2+}$ coordination geometry. In bidentate hydroxamate complexes with HDAC6, the hydroxamate NH group donates a hydrogen bond to H574.48,142,159 The same is true in human HDAC8–hydroxamate inhibitor complexes, where the hydroxamate NH group donates a hydrogen bond to H143.51,72 Thus, the mercaptoaacetamide moiety is, in effect, a functional mimic of a hydroxamate group in terms of its ability to make an identical constellation of metal coordination and hydrogen bond interactions in the HDAC6 active site. Even better, the mercaptoaacetamide moiety is not mutagenic since it is not subject to degradation via the Lossen rearrangement.

Why, then, does the mercaptoaacetamide exhibit different binding interactions in the active site of SmHDAC8, lacking a hydrogen bond with the second active site histidine in the tandem pair? The mercaptoaacetamide moiety clearly does not serve as a functional mimic of a hydroxamate group in binding to HDAC8. This difference likely contributes to weaker inhibition of class I HDAC isozymes by mercaptoaacetamides.154,155 Structural comparisons of HDAC6, SmHDAC8, and human HDAC8 reveal that the second histidine is in a different electrostatic environment in each isozyme. In SmHDAC8 and human HDAC8, this histidine (H142 in SmHDAC8, H143 in human HDAC8) donates a hydrogen bond to a negatively charged carboxylate side chain (D191 in SmHDAC8, D183 in human HDAC8), which elevates the histidine pK$_a$.77 In contrast, the corresponding histidine in HDAC6, H574, donates a hydrogen bond to the neutral carboxamide side chain of N617, which would elevate the pK$_a$ of H574, but not as much as would result if the hydrogen bond were made with a negatively charged carboxylate. Accordingly, H574 of HDAC6 is less basic than H142/H143 of SmHDAC8/human HDAC8. Consequently, H142 of
$\Delta m$HDAC8 and H143 of human HDAC8 are more likely to be protonated than H574 of HDAC6 at a given pH.

Differences in the basicity of the second histidine in the tandem pair also have implications for catalysis. In human HDAC8, enzymological and structural studies indicate that the second histidine, H143, functions as a single general base–general acid.\textsuperscript{77} Although similar enzymological studies have not yet been performed with HDAC6, deletion of the second histidine by mutagenesis in the H574A variant enables the crystallization and structure determination of an intact enzyme–substrate complex as the tetrahedral intermediate in the catalytic mechanism.\textsuperscript{48} This observation implies that the general base functionality in the active site of H574A HDAC6 is preserved – formation of the tetrahedral intermediate requires a sufficiently nucleophilic water molecule activated by Zn\textsuperscript{2+} coordination and a general base. Thus, the remaining active site histidine, H573 in H574A HDAC6, might function as the general base in this isozyme. H574 must serve as the general acid, since its deletion leads to the trapped intermediate. The tetrahedral intermediate of amide hydrolysis cannot collapse without a proton donor to the leaving amino group, so the deletion of the general acid in H574A HDAC6 results in the formation of the tetrahedral intermediate as a dead-end complex.

Other aspects of MCA binding to HDAC6 contribute to its selectivity as well. The aliphatic linker packs into the aromatic groove in the substrate binding cleft with distances of 3.7 Å and 3.5 Å to the phenyl rings of F583 and F643 respectively. The capping group of the inhibitor is situated within the previously characterized L1 loop pocket, an interaction that confers HDAC6 selectivity.\textsuperscript{159} The chlorine atoms of the dichloroindole capping group pack against the side chains of H463 and P464. Additionally, the capping group of the inhibitor bound to monomer A packs
against R636, D638, and F642 of monomer B. Meanwhile, the molecule bound to monomer B forms lattice contacts against D460 and H462 of monomer A.

It is worthwhile to note that thiol-containing drug candidates such as MCA can be subject to oxidation chemistry or reaction with an unintended electrophile \textit{in vivo}, which could compromise their inhibitory function. The thiol-containing HDAC inhibitors Romidepsin and Largazole evolved to exist as thioester and disulfide-linked prodrugs, respectively.\textsuperscript{37} Romidepsin is activated by reduction of its internal disulfide linkage to yield the active inhibitor Romidepsin thiol, and Largazole is activated by hydrolysis of its thioester linkage to yield Largazole thiol. That being said, HDAC6 is localized in the cell cytosol,\textsuperscript{122} which is highly reducing, so this would favor the free thiol form of such inhibitors, including MCA. Moreover, there is precedent for the efficacy of a thiol-functionalized drug as exemplified by Captopril, which contains a thiol group targeting Zn\textsuperscript{2+} coordination in the active site of angiotensin converting enzyme.\textsuperscript{160,161}
6.4 | Conclusion

In summary, the present study highlights a chemical difference in the binding of mercaptoacetamides and hydroxamates to HDAC6 and HDAC8, specifically with regard to interactions with the tandem histidine pair in the active site. While each class of inhibitor contains a functional group that directly coordinates to Zn$^{2+}$ (C-S$^{-}$ and N-O$^{-}$, respectively), interactions with nearby active site residues differ. When bound to either enzyme, the hydroxamate N-O$^{-}$ group interacts with both histidine side chains. However, mercaptoacetamides exhibit different interactions in HDAC6 and HDAC8, specifically with regard to the second histidine. This highlights the importance of the tandem histidine pair in each of these enzymes – differences in the basicity of the second histidine residue can influence inhibitor binding and catalysis, which in turn can be exploited to enhance inhibitor selectivity.
Chapter 7 | Summary & Prospects

Summary

Taken as a whole, the works described in this thesis have advanced our understanding of the chemical principles underlying isozyme-selective HDAC inhibition. Discovery of the unusual monodentate binding mode exploited by potent HDAC6 inhibitors highlights the shallower nature of this enzyme's active site which can be targeted by employing sterically bulky capping groups. One such region of the enzyme surface is a cavity formed by the L1 loop, which is more recessed from the active site than in other isozymes and readily accommodates nonpolar moieties. Since the entropy of inhibitor binding is generally positive for HDAC6 while the same value is negative for HDAC8, it appears that HDAC6 is predisposed to be targeted by simple hydroxamate inhibitors. Characterization of the entropic change upon the binding of future drug leads will help to identify scaffolds that best take advantage of this phenomenon. Additionally, differences in the hydrogen bonding network for mercaptoacetamide inhibitors bound to HDAC6 and HDAC8 highlight subtle mechanistic differences that may be exploitable in the engineering of better-targeted therapeutics. All in all, this work has contributed to the foundation upon which future efforts toward the selective inhibition of HDACs can be built.

Future Directions

Major challenges still exist in pursuit of selective HDAC inhibition. The pursuit of potent and selective nonhydroxamate zinc binding groups is vital to the development of therapeutics that are amenable to long-term treatments. While mercaptoacetamide-based inhibitors discussed here have certainly been shown to be neuroprotective in a mouse model,\textsuperscript{155} the thiol zinc binding group suffers from certain challenges due to its nucleophilicity and susceptibility to redox chemistry within
the cell. As a result, further exploration of potential inhibitory warheads is required. With the discovery that irreversible class I HDAC inhibition by α,β-epoxyketones (such as that found in trapoxin A) is dependent on steric rather than a long-presumed covalent modification, it is possible that α,β-cyclopropyl- or cyclobutylketones may serve as viable pharmacophores. The privileged status of cyclopropyl groups in many pharmaceuticals makes this a particularly attractive line of inquiry. Additionally, since HDAC inhibitors are currently used in the clinic to sensitize cancers to other therapeutics, the design of dual-faced inhibitors (i.e. a single-molecule that inhibits multiple pathways in combinatorial therapies) may assist in the development of inhibitors for the treatment of specific conditions in which multiple biological processes have become dysregulated. The validity of this concept has already been demonstrated in the design of RTS-V5, a phenylhydroxamate-based HDAC6 inhibitor bearing a proteasome inhibitor as its capping group.

With regard to HDAC function, one of the largest open questions concerns the substrate specificity of particular isozymes. In other words, how is the deacetylation of almost 40,000 known acetylation sites regulated by less than 20 enzymes? While it is likely that there are enzymes capable of lysine deacetylation that remain uncharacterized, it has still been shown that many of the metal-dependent HDACs are capable of deacetylating several different substrate proteins. It follows that factors influencing isozyme selective inhibition may also play a role in substrate selection by a particular HDAC. This is evidenced when comparing the structures of the HDAC8-trapoxin A complex, in which an aspartate mimics substrate binding through two hydrogen bonds to the backbone around the scissile acetyllysine, and the HDAC6-HC toxin complex, in which an isosteric serine makes only a hydrogen bond. This suggests that HDAC6 can better accommodate acetyllysine substrate immediately followed by a proline residue, while HDAC8 would require a nonproline
residue in this position. Therefore, the determination of structures with other peptidomimetic inhibitors will provide information about both inhibition and catalysis in HDAC enzymes.

To better understand the structural underpinnings of HDAC substrate specificity, it is also vital to determine crystal structures between catalytically-inactive HDACs and substrate peptides or proteins. An ideal starting place for this work is the complex between HDAC8 and its most well-characterized substrate, structural maintenance of chromosomes protein 3 (SMC3). The structure of the yeast ortholog of SMC3 has been determined, providing a good starting point for the design of a crystallizable construct of the human protein. Due to its broad range of substrates, a complex between HDAC8 and a protein substrate would provide valuable insight into the interactions required for identification of a substrate for deacetylation. The general conservation of the active site across most metal-dependent HDACs suggests that substrates are selected by interactions at the enzymes surface, rather than at the site of deacetylation. The characterization of specific HDAC-substrate pairs may even provide access to pharmaceuticals targeting specific pathways of deacetylation. This would represent a major advance in understanding the regulatory pathways employed at the cellular level for the maintenance of life’s processes.
Bibliography


(60) Kaiser, F. J.; Ansari, M.; Braunholz, D.; Gil-Rodríguez, M. C.; Decroos, C.; Wilde, J. J.;


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42–48.


