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A bioinorganic approach to fragment-based drug discovery targeting metalloenzymes

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CONSPECTUS

Metal-dependent enzymes (i.e. metalloenzymes) make up a large fraction of all enzymes and are critically important in a wide range of biological processes, including DNA modification, protein homeostasis, antibiotic resistance, and many others. Consequently, metalloenzymes represent a vast, and largely untapped space for drug development. The discovery of effective therapeutics that target metalloenzymes lies squarely at the interface of bioinorganic and medicinal chemistry and requires expertise, methods, and strategies from both fields to mount an effective campaign. In this Account, our effort to bring together the principles and methods of bioinorganic with those of medicinal chemistry to bridge the gap between these fields and address an important class of medicinal targets are described.

Fragment-based drug discovery (FBDD) is an important drug discovery approach that is particularly well suited for metalloenzyme inhibitor development. FBDD uses relatively small, but highly targeted chemical structures that allow for the assembly of privileged molecular collections that focus on a specific feature of the target enzyme. For metalloenzyme inhibition the specific feature is rather obvious, namely a metal-dependent active site. Surprisingly, prior to our work the exploration of diverse molecular fragments for binding the metal active sites of metalloenzymes was largely unexplored. By assembling a modest library of metal-binding pharmacophores (MBPs), we have been able to find lead hits for many metalloenzymes, and from these hits develop inhibitors that act via novel mechanisms of action. A specific case study on the use of this strategy to identify a first-in-class inhibitor of zinc-dependent Rpn11 (a component of the proteasome) is highlighted.

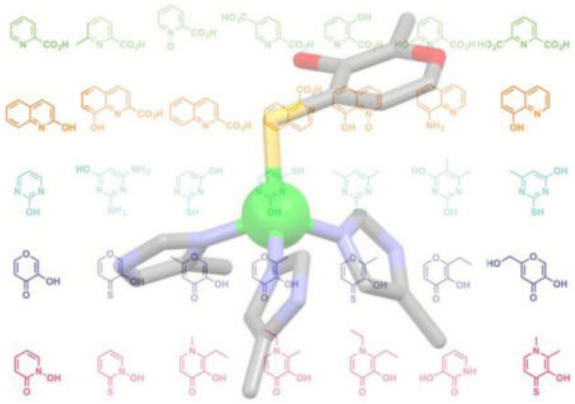
The application of FBDD for the development of metalloenzyme inhibitors has raised several other compelling questions, such as how the metalloenzyme active site influences the coordination chemistry of these bound fragments, and how one can identify the best fragments for a given metalloenzyme, and many others. Among the most significant, and concerning, questions for metalloenzyme inhibition reside around the question of specificity and whether metalloenzyme inhibitors can be as selective and specific as other small molecule inhibitors (i.e., compounds that inhibit enzymes that do not utilize a metal at their active site). This also leads to the question of whether metalloenzyme inhibitors might interfere more broadly with the metallome. Efforts to

BIOGRAPHICAL INFORMATION

Seth M. Cohen received a B.S. in Chemistry and B.A. in Political Science from Stanford University in 1994. He completed his Ph.D. in Chemistry at U.C. Berkeley under the direction of Prof. Kenneth N. Raymond and performed postdoctoral studies with Prof. Stephen J. Lippard at M.I.T. He started his independent career at U.C. San Diego in 2001.

address these and related questions are discussed, with the expectation that our findings will answer some of these questions, alleviate some of these concerns, and encourage greater interest in this important, undervalued class of drug targets.

Graphical Abstract



Metalloenzymes make up more than 30% of all known enzymes. Most small molecule drugs target enzymes to elicit their therapeutic effect. Taken together, these facts suggest that roughly one-third of all drug should target metalloenzymes; however, a recent review suggests that less than 70 FDA approved drugs are metalloenzyme inhibitors, targeting only 7 classes of metalloenzymes.[1] With nearly 1500 FDA approved drugs, this indicates that less than 5% of small molecule drugs target a metalloenzyme. This large discrepancy between the number of potential metalloenzyme targets (>30% of target space) and the number of drugs developed for these targets (<5% of drug approvals) indicates a knowledge gap in developing metalloenzyme inhibitors, but also an opportunity for bioinorganic chemists to help fill this gap.

As their broad use in nature suggests, metalloenzymes are critical to nearly all biological processes and all living organisms, and as such represent a rich target space for drug development for a wide variety of diseases. Some representative examples of clinically approved metalloenzyme inhibitors are shown in Figure 1, which as anticipated, highlights the wide spectrum of diseases that these drugs address including hypertension, glaucoma, fungal infections, cancer, and HIV/AIDS.[2] All of these drugs utilize direct coordinate covalent binding to the active site metal ion to inhibit their targets.

The functional groups used in these inhibitors to bind to the active site metal ion have been given different names in the literature including chelator, zinc-binding group (ZBG, for Zn-dependent metalloenzyme inhibitors), and metal-binding group (MBG); however, in this manuscript the term metal-binding pharmacophore (MBP) will be employed, which represents a deliberate effort to merge the vernacular of inorganic and medicinal chemists. A major shortcoming in the field of metalloenzyme inhibitors is the rather limited number of MBPs that have been employed to bind the active site metal ion. The narrow exploration of MBP chemical space is perhaps best illustrated by the considerable efforts made to develop inhibitors of matrix metalloproteinases (MMPs), a class of Zn-dependent metalloenzymes

involved in a wide range of biological processes. For nearly 20 years, beginning in the 1980s, MMPs were a prominent metalloenzyme target for the development of arthritis and cancer therapeutics. A comprehensive review by Whittaker in 1999 highlighted several compounds that had been developed up to that time,[3] which revealed the very apparent reliance on a single MBP – the hydroxamic acid functional group (Figure 2). The hydroxamic acid functional group is found in natural products such as siderophores,[4] molecules used by microorganisms to acquire iron from their environment. Even though the hydroxamic acid MBP was prominently featured in natural products used to sequester Fe(III), it was also the nearly exclusive MBP used to inhibit the zinc-dependent MMPs.[3] Indeed, it could be argued that the hydroxamic acid MBP was used as a 'silver bullet', not only for MMPs, but in many subsequent drug discovery efforts to identify other metalloenzyme inhibitors. For example, efforts spanning more than a decade by pharmaceutical companies to develop inhibitors of Zn-dependent LpxC (found in Gram negative bacteria) all utilized the hydroxamic acid MBP (Figure 2). The nearly singular reliance on the hydroxamic acid MBP becomes even more perplexing considering that: a) the MBP is not selective for Zn(II) over other metal ions; b) the MBP is known to have pharmacokinetic (PK) liabilities (e.g. poor in vivo stability); and c) few metalloenzyme inhibitors that employ the hydroxamic acid MBP are clinically approved. It was this nearly exclusive, and somewhat inexplicable, use of the hydroxamic acid MBP that inspired the author to seek alternative, bioinorganic approaches to drugging metalloenzymes.

As a final point of emphasis, the near-exclusive use of the hydroxamic acid MBP for binding the active site metal ion of metalloenzymes was, by analogy, akin to requiring every drug discovery effort that targeted a hydrophobic pocket to use only an indole heterocycle. Any medicinal chemist would view such an arbitrary and restrictive design criterion as nonsensical with respect to the available chemical space. By analogy, the bioinorganic chemist should view the singular use of the hydroxamic acid MBP as similarly unreasonable. With this premise in mind, our group has introduced alternative MBPs for the development of metalloenzyme inhibitors. In doing so, we have linked medicinal and bioinorganic chemistry, as well as generated findings in bioinorganic coordination chemistry that provide insight into the binding of small molecules to metalloenzyme active sites and the relation back to metalloenzyme drug discovery.

'Debunking' the Hydroxamic Acid Myth

To introduce viable alternatives to the hydroxamic acid MBP it was necessary to convince those interested in metalloenzyme inhibitors that the hydroxamic acid could be 'beat' and did not constitute a 'silver bullet'. To do this, it was necessary to take an existing metalloenzyme inhibitor and show that substitution of a hydroxamic acid for an alternative MBP could lead to an inhibitor with improved activity. This was a first step in establishing a viable program that would capture the attention of the medicinal chemistry community. Because of the substantial amount of work on MMP inhibitors and their reliance on the hydroxamic MBP,[3] MMPs were selected for our initial investigations. Our goal was to take a known MMP-3 (stromelysin) inhibitor reported by Fesik[5] (compound **SF-3**, Figure 3, discovered coincidentally by fragment-based drug discovery) and make a single atom substitution, converting the hydroxamic acid MBP into a thiohydroxamic acid MBP (**SF-3S**,

Figure 3), thereby exploiting the intermediate hard-soft acidity of the Zn(II) ion at the MMP active site. Existing solution thermodynamics data on hydroxamic acid versus thiohydroxamic acid binding to the Zn(II) ion suggested that this single atom change should result in a greater than 10-fold improvement in activity. Such a significant improvement in inhibitor binding by simply changing a MBP donor atom from oxygen to sulfur would demonstrate that there were viable alternatives to the hydroxamic acid MBP.

Alas, synthetic challenges, poor solubility, and questionable stability of the thiohydroxamic acid precluded this demonstration of alternative MBPs. However, we were inspired to explore related derivatives that could act as alternative MBP fragments. A small collection of approximately a dozen MBPs, including acetohydroxamic acid (**AHA**, Figure 3) as a hydroxamic acid surrogate, was assembled and tested for inhibitory activity against MMP-3. The alternative MBPs (**1**-**11**, Figure 3) were expected to be more active than acetohydroxamic acid based on several features, including conformation rigidity, greater acidity (e.g. lower pK_a, when compared to hydroxamic acid), and use of a preferred mixed hard-soft (*O,S*) donor atom set. Screening of these MBPs against MMP-3 was performed using routine enzymatic assays.[6] This illustrates one of the advantages of MBP fragments, when compared to conventional fragments, where the tighter binding of MBPs to metalloenzymes (because of the formation of relatively strong coordinate covalent bonds to the metal ion) allows for screening via conventional in vitro assays, rather than more sophisticated screening technologies (vide infra).[7,8] Importantly, screening of this small MBP collection demonstrated that our chemical intuition was correct, as all the alternative MBPs were more active against MMP-3 than acetohydroxamic acid (Figure 3). Furthermore, when one of these MBPs, maltol (3-hydroxy-2-methyl-4*H*-pyran-4-one, compound **5**, Figure 3), was used to replace the hydroxamic acid MBP in **SF-3**, it was shown to produce an analog with better activity (**AM-5**, Figure 3). Maltol is an approved flavor additive (as it possesses cotton candy-like smell) suggesting it should have different, and potentially better, PK properties than a hydroxamic acid. This improved activity, coupled with the possibility of better biocompatibility and metabolism characteristics, convinced us that the hydroxamic acid should no longer be considered a privileged scaffold for metalloenzyme inhibition.

MBP Libraries for FBDD

Our initial reports looked at only a handful of MBP compounds (Figure 3) to challenge the supremacy of the hydroxamic acid moiety. Having demonstrated that other MBPs could display superior activity,[6,9] our attention turned to examining other metal-binding motifs that could serve as MBPs. Focused primarily on inhibiting Zn-dependent metalloenzymes, we explored nitrogen-rich ligands that were commonly found in the coordination chemistry literature of Zn(II). Much of our inspiration came from studies of molecular sensors for Zn(II), adopting the ‘receptor’ portion of these molecules, which included polypyridyl ligands, aza macrocycles, and quinolone sulfonamides.[10,11] Nevertheless, our work remained largely confined to a small number of molecules (~20 MBPs) until we were introduced to the concept of fragment-based drug discovery (FBDD, also referred to as fragment-based lead discovery, FBLD).[8]

FBDD was an emerging drug discovery strategy in the late 1990s and early 2000s that involved exploring libraries of molecular ‘fragments’.[7,8] Fragments were distinguished from traditional compound libraries primarily by their small molecular weight. Upon identifying a fragment that binds with suitable ligand efficiency to a target, then strategies, including fragment growth, linking, merging, and tethering could be used to transform these fragment hits into complete, drug-like compounds.[7] Analogous to the often quoted Lipinski ‘Rule-of-Five’ for drug-likeness,[12] Congreve et al. devised a ‘Rule-of-Three’ for fragments suitable for library development, namely:[7,13] molecular weight <300, hydrogen bond donors ≤3, hydrogen bond acceptors ≤3, and ClogP ≤3.

Upon attending drug discovery conferences and workshops, and increased interactions with medicinal chemists, it eventually became apparent to the author that the development of new MBPs was an exercise in FBDD. Indeed, among the early, pioneering studies in FBDD, Fesik et al. had applied FBDD to MMP-3 to identify **SF-3** (Figure 3).[5] However, in this study, only the hydrophobic fragment of the inhibitor was varied, while the MBP of the inhibitor was kept constant as acetohydroxamic acid (**AHA**, Figure 3). In a subsequent study, Fesik et al. did perform a limited FBDD examination of MMP-3 using some conservative changes to identify MBPs with better PK than the hydroxamic acid.[14] The result of this study was the selection of a 1-naphtyl hydroxamic acid as an improved fragment, which is not a new MBP, but rather simply a substituted hydroxamic acid that demonstrates improved PK properties. Although 1-naphtyl hydroxamic was quite active against MMP-3 (IC_{50} value = 50 μM), use of this fragment in a complete inhibitor resulted in a loss (~6-fold) in activity when compared with **SF-3**. Only with considerable changes in the linkage between the MBP and the biaryl ‘backbone’ was activity comparable to **SF-3** restored.[14]

It became obvious that applying FBDD to metalloprotein inhibitors had several potential advantages over conventional FBDD methods. Generally, FBDD uses fragments that interact with target proteins via weak interactions (e.g., hydrophobic, H-bonding, etc.) and hence fragments often possess low binding affinities and ligand efficiencies. Due to their weak affinities, binding of these fragments often cannot be detected and quantitated using conventional assays or screening methods, such as in vitro biochemical assays. Consequently, more sophisticated biophysical methods, such as surface plasmon resonance (SPR) or X-ray crystallography are required to detect binding. The non-specific binding of conventional fragments necessitates the use of macromolecular X-ray crystallography or NMR methods to determine where a fragment is binding on a target. In contrast, a designed library of MBPs can be largely relied on to bind at the active site metal ion. This leads to an additional advantage of an MBP library, which is that the rich field of inorganic/bioinorganic spectroscopy can also be used to detect and elucidate the details of MBP binding to the metalloenzyme active site. Methods such as X-ray absorption spectroscopy (XAS, including EXAFS), electron paramagnetic resonance (EPR) spectroscopy, and Mössbauer spectroscopy can all be applied to examining MBP binding and are largely not applicable to the binding of typical FBDD fragments. Similarly, in the absence of structural biology, bioinorganic model complexes[15,16] can serve to reveal possible and probable modes of MBP binding to guide computational drug discovery efforts.[9,17] Finally, MBP libraries

can be rationally assembled, not only for metalloenzymes generally, but can be enriched to favor certain subclasses of metalloenzymes, such as dinuclear metalloenzymes or heme metalloenzymes. Therefore, the development of MBP-based FBDD libraries for metalloenzymes has many attractive features. To quote Silverman,[18] "*One strategy that can be used for potentially more effective libraries is to select privileged structures as the scaffold*" and "*Another strategy is to design a scaffold based on an important molecular recognition motif in the target receptor*." With MBP libraries we sought to utilize both strategies to achieve greater success in drugging metalloenzyme targets.

The first MBP fragment libraries were reported in 2010 and 2011.[19,20] These libraries were originally termed 'CFL' for 'chelator fragment library', but we have since changed our terminology (from chelator to MBP) to avoid the negative connotations the word 'chelator' carries in the medicinal chemistry community. The library consisted of 96 fragments (for screening in 96-well plates) and contained several families of MBPs including picolinic acids, quinolines, pyrimidines, hydroxypyrones, hydroxypyridinones, salicylic acids, and other miscellaneous metal-binding fragments (Figure 4). In these initial reports, the CFL library was screened against nine metalloenzymes: MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, anthrax lethal factor (LF, zinc-dependent), 5-lipoxygenase (5-LO, non-heme iron-dependent), tyrosinase (TY, dinuclear copper-dependent), and inducible nitric oxide synthase (iNOS, heme iron-dependent). The results of this screening campaign revealed several important features that validated the use of MBP fragment libraries for FBDD: 1) as alluded to earlier, standard in vitro enzyme-based assays could be used to screen the library (precluding the need for more sophisticated screening techniques); 2) very few MBP fragments inhibited all nine metalloenzymes demonstrating selectivity of certain MBPs for given metalloenzymes; 3) some subsets of MBPs were preferred by different metalloenzymes (i.e. demonstrating a rudimentary structure-activity relationship, SAR); and 4) essentially none of the 96 MBPs inhibited iNOS, which was by design, as the fragments in the CFL library were chosen to be most effective against non-heme enzymes. This latter element was achieved by selecting MBPs that could bind to at least two adjacent sites on the coordination sphere of the active site metal ion (i.e., act as a bidentate chelating ligand), which is not accessible for a heme active site. Collectively, these initial findings validated many of the fundamental features and feasibility of using MBPs and FBDD for the development of metalloenzyme inhibitors.

Vindication by Validation: New Metalloprotein Inhibitors

Having developed a MBP library that could serve as a platform for metalloenzyme inhibitor discovery, it became critical to demonstrate that this library could lead to the development of novel, active metalloenzyme inhibitors. Since the first complete report on the CFL in 2011, [20] our laboratory has developed inhibitors against a wide variety of metalloenzyme targets. [9,11,21,22] In most cases, these inhibitors have been developed by using a fragment growth strategy[7,8] and in many instances represent the first non-hydroxamate inhibitor of a given metalloenzyme, the most active non-hydroxamate inhibitor of a given metalloenzyme, or an altogether first-in-class inhibitor. The discovery of first-in-class small molecule inhibitors best shows the importance of our approach, and hence one such case study will be presented on the zinc-dependent proteinase Rpn11 found within the proteasome.

The ubiquitin−proteasome system (UPS) degrades unwanted proteins, can influence cellular processes (i.e., cell cycle, apoptosis), and has become a prominent target in recent years for the treatment of cancer.[23] The proteasome is composed of a 20S core particle (20S CP) and a 19S regulatory particle (19S RP).[24] Proteasome inhibitors (i.e. bortezomib, carfilzomib, ixazomib) that are currently used for the treatment of cancer target the primary site of proteolysis within the 20S CP. In contrast, Rpn11 is a Zn-dependent proteinase that resides within in the 19S RP.[25] The Rpn11 subunit cleaves ubiquitin from the proteins to be proteolyzed within the 20S CP; interfering with Rpn11 activity prevents cleavage of the ubiquitin tag,[26] and hence blocks proteolytic degradation of the tagged substrate as it cannot access the 20S CP with the ubiquitin tag still intact. Therefore, inhibition of Rpn11 has been proposed as another means of inhibiting the proteasome for new anticancer therapeutics.

Despite substantial efforts in both academia and industry, no small molecule inhibitors of Rpn11 had been described prior to our work. In collaboration with Raymond Deshaies and co-workers,[22,27] we screened the CFL library against Rpn11. Indeed, a biochemical screen of ~100 compounds revealed a single, highly active hit 8-thioquinoline (**8TQ**, Figure 5).[22] This screening campaign was somewhat unique in two ways: first, the screen identified only **8TQ** as an active MBP, which was unusual as generally more than one active MBP would be found in our screening campaigns against other metalloenzymes.[21] Second, this hit was exceptionally active, with an IC_{50} value between ~2 and 3 μM, affording this small compound an outstanding ligand efficiency of 0.69.[7] An important observation is that an earlier, high-throughput screen (HTS) of compounds from the National Institutes of Health Molecular Libraries Small-Molecule Repository against Rpn11 revealed only one validated hit, which proved to be a thioester derivative of **8TQ**.[27] The contrast between the modest size of our FBDD library (~100 compounds) and the HTS screen (~330,000 compounds) shows the efficiency and effectiveness of using a highly targeted, rationally designed compound library.

Upon identifying **8TQ** as a hit, a small SAR study was performed to: a) identify suitable points of derivatization to increase **8TQ** activity and selectivity, and b) confirm the metal-binding core pharmacophore of **8TQ**.[22] To identify positions on the **8TQ** that would tolerate substitution, several methylated derivatives of **8TQ** were prepared. These analogs showed that 3-methyl and 4-methyl-8-thioquinoline retained activity against Rpn11 comparable to unsubstituted **8TQ**. To confirm the core pharmacophore of **8TQ**, several close analogues were prepared, all of which have some impairment in metal binding (Figure 5). Supportive of a metal-coordinating mode of inhibition, all of these analogs were inactive against Rpn11. In contrast, a heterocyclic analog of **8TQ** (**TQ-D8**, Figure 5) retained activity with an IC_{50} value of ~15 uM, consistent with the proposed mode of action. A possible mode of binding was suggested by a bioinorganic model of the Rpn11 active site in the form of a [($Tp^{Me,Ph}$)Zn(**8TQ**)] ($Tp^{Me,Ph}$ = hydrotris(5,3-methylphenylpyrazolyl)borate) complex.[22] As shown in Figure 6, the model complex shows that **8TQ** binds to the Zn(II) ion in the expected bidentate manner using the nitrogen and sulfur donor atoms of the ligand. This is wholly consistent with the inhibition data of the analogs described above,

where moving, removing, replacing, or blocking any of these donor atoms results in a loss of activity.

With the basic SAR in hand, a fragment growth strategy using 3-carboxy-8-thioquinoline as a common intermediate was initiated. Several dozen 3-carboxyamide derivatives were synthesized and a SAR emerged suggesting that heterocyclic amines gave the most active and selective Rpn11 inhibitiors.[22] Upon optimizing the spacer length between the MBP and the heterocycle an inhibitor given the name ‘capzimin’ was identified.[27] Capzimin has an IC_{50} value of 340 nM against Rpn11 and shows good selectivity over related Zn-dependent proteases. More importantly, capzimin induced accumulation of polyubiquitinated substrates (consistent with proteasome inhibition) and showed antiproliferative activity against several cancer cell lines, was confirmed to induce apoptosis in some of these cell lines, and showed activity against cell lines that were resistant against proteasome inhibitors that target the 20S CP (consistent with a novel mechanism of proteasome inhibition, i.e., Rpn11 inhibition in the 19S RP).[27] This is just one of many case studies of how FBDD using an MBP library has been used to successfully identify a novel metalloenzyme inhibitor. Capzimin is particularly notable as large scale HTS failed to produce viable lead compounds, but our FBDD approach resulted in a first-in-class inhibitor of the high value target Rpn11.

Determining Metalloprotein Inhibitor Selectivity

Despite the clinical success of several metalloenzyme inhibitors, there appears to be a lingering bias against the development of these therapeutics. This bias originates from a perception that any compound that contains an MBP will be inherently non-selective and result in either off-target metalloenzyme inhibition, disruption of normal metal ion homeostasis, or both. This prejudice exists despite little literature evidence to support such claims. Therefore, in addition to our work on developing ‘new and improved’ metalloenzyme inhibitors, we have also performed studies to evaluate whether metalloenzyme inhibitors, as a broad class of therapeutics, show a greater propensity for off-target effects because of their molecular mechanism of action, namely binding to and blocking of coordination sites on an active site metal ion.

To test the hypothesis that metalloenzyme inhibitors were poorly selective, a panel of metalloenzymes and their respective inhibitors were evaluated in cross-inhibition assays.[28] Figure 7 shows a list of the metalloenzyme inhibitors tested as well as their respective metalloenzyme targets. The inhibitors examined cover a wide range of chemical structures and MBP motifs, including sulfonamides (acetazolamide), thiols (captopril), hydroxamic acids (SAHA, CGS), and several others. The metalloenzymes examined covered a similarly broad range of targets including zinc-, manganese-, and iron-based enzymes involved in pathologies ranging from hypertension to HIV/AIDS. This panel of inhibitors were screened against all the target enzymes these compounds collectively inhibited, namely, carbonic anhydrase (hCAII), several matrix metalloproteinases (MMPs), angiotensin converting enzyme (ACE), histone deacetylase (HDAC-2), and tyrosinase (TY), as well as a non-metalloenzyme target trypsin. Although these inhibitors all possess IC_{50} values against their targets in the 10-600 nM range, none of these compounds showed significant activity against

non-target enzymes at concentrations as high as 10 μM.[28] For example, as shown in Figure 8, at a concentration of 10 μM only acetazolamide shows inhibition of hCAII (the target of acetazolamide). Although the other compounds tested had a putative MBP, only acetazolamide shows any activity against hCAII. In addition to low or no activity against off-target metalloenzymes, none of the inhibitors showed activity against trypsin (which is not metal dependent). Collectively, these data clearly suggest that these metalloenzyme inhibitors are no less selective than other enzyme inhibitors. In a related set of experiments, we examined the ability of a subset of these same metalloenzyme inhibitors to selectively inhibit their intended target in a medium containing a large excess of one or more off-target metalloproteins.[29] Metallothionein, myoglobin, carbonic anhydrase, and transferrin were selected as competing metalloprotein based on their availability and their diversity of metal sites. The use of competing metalloproteins in these experiments was designed to parallel the common use of human (HSA) or bovine (BSA) serum albumin in enzyme assays to evaluate non-specific binding to plasma proteins. A common approach to evaluating non-specific plasma protein binding for small molecule inhibitors is to add HSA or BSA to the assay medium and monitor any loss in inhibitor activity, which is indicative of non-specific, off-target protein binding to HSA or BSA that results in a lower effective inhibitor concentration. In our experiments, the four competing metalloproteins listed above play a similar role by providing off-target metal sites that can titrate away potentially lower the effective concentration of metalloenzyme inhibitor in the assay. Ultimately, experiments using these competing proteins led to the same conclusion; even when all four competing metalloproteins were present at >400-fold concentration over the target metalloenzyme,[29] no change in inhibitor activity was noted, supporting the case for a high degree of selectivity by metalloenzyme inhibitors.

The aforementioned experiments were focused on evaluating the selectivity of metalloenzyme inhibitors with respect to off-target inhibition. A separate, but related concern, centers around the possibility of metalloenzyme inhibitors interfering with metal ion homeostasis, trafficking, and metabolism. To evaluate this issue, two sets of experiments have been performed: first, examining the ability of metalloenzyme inhibitors to remove iron from transferrin; and second, the effect of sublethal concentrations of histone deacetylase (HDAC) inhibitors on the metal ion distribution and content of mammalian cells. In the first set of experiments, the ability of the inhibitors listed in Figure 7 to remove iron from holotransferrin was evaluated. Even at concentrations of 1 mM, none of the compounds showed any iron removal except for 1,2-HOPO-2 (Figure 7), which showed some activity, but far below that of the bacterial siderophore desferroxamine (a FDA-approved iron chelator, Figure 7).[28] As transferrin represents among the most accessible transition metal pools in humans, and as such are the targets of bacterial siderophores (e.g. desferroxamine, Figure 7), these data suggest that metalloprotein inhibitors are incapable of disrupting metal ion homeostasis, at least via this trafficking pathway.

In the second set of experiments, three different HDAC inhibitors (Figure 9) were examined for their propensity to alter the metal ion content or distribution in the mouse fibroblast NIH3T3 cell line.[30] The three HDAC inhibitors were selected because they share a common metalloenzyme target (i.e. zinc-dependent HDACs), but possess different MBPs with vastly different metal-binding affinities. Therefore, the aim was to be able to distinguish

between changes in metal ion metabolism as a function of HDAC inhibition (which all the inhibitors should display) versus that caused by a greater affinity of one of the inhibitors to bind metal ions. Due to the difficulty of these experiments a combination of methods, including inductively coupled plasma atomic emission spectroscopy (ICP-OES), energy-dispersive X-ray spectroscopy (EDX), and synchrotron X-ray fluorescence microscopy (SXRF) were utilized. ICP-OES was used to monitor the whole cell metal content, while SXRF was used to look for changes in cellular metal ion distribution, and EDX was used to examine both overall content and changes in the distribution of one metal ion (Zn). Ultimately, within the limits of these techniques, no prominent changes in metal ion content or distribution were observed upon treatment with sublethal concentrations of HDAC inhibitors (Figure 9).[30] These results, collectively with the other findings described above, lead us to the same conclusion we reached in 2013,[28] which is that metalloenzyme inhibitors do not pose any greater risk for off-target activity than any other class of small molecule enzyme inhibitors.

Conclusions and Outlook

Bringing the knowledge and knowhow of bioinorganic chemistry to address a substantial issue in medicinal chemistry has been a productive and exciting area of research for the author's laboratory for the last 15 years. Merging bioinorganic and medicinal chemistry and learning about how these disciplines both complement and inform each other has revealed new science and concepts that enhance the arsenal of tools available to tackle metalloenzyme targets. With an initial goal of simply demonstrating alternatives to the ubiquitous and overworked hydroxamic acid ligand, our blending and understanding of bioinorganic and medicinal chemistry concepts has led to fragment libraries of MBPs, a deeper understanding of coordination chemistry within metalloenzyme active sites,[31-34] and attempts to address questions around inhibitor target and metallome specificity. Tremendous recent successes of metalloenzyme inhibitors (e.g. HIV integrase inhibitors) indicate that these therapeutics will be needed to address challenges in antivirals, antineoplastics, antibiotics, and systemic illnesses that become more common in a longer-lived population. The use of FBDD to identify metalloenzyme inhibitors has continued to grow,[35-38] and it is hoped that the insight provided by our efforts and those of our colleagues across the globe, will help to make discoveries that will advance not only understanding in bioinorganic and medicinal chemistry, but contribute to finding solutions to problems that are of great value to human health.

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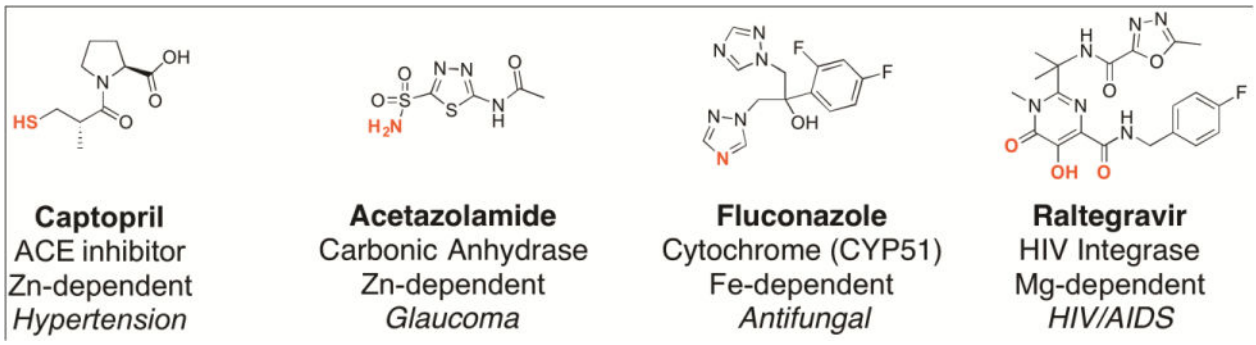

Figure 1.

Examples of several FDA approved metalloenzyme inhibitors with the name, target, active site metal ion, and indication for each compound listed. Donor atoms that bind the active site metal ion are shown in bold red.

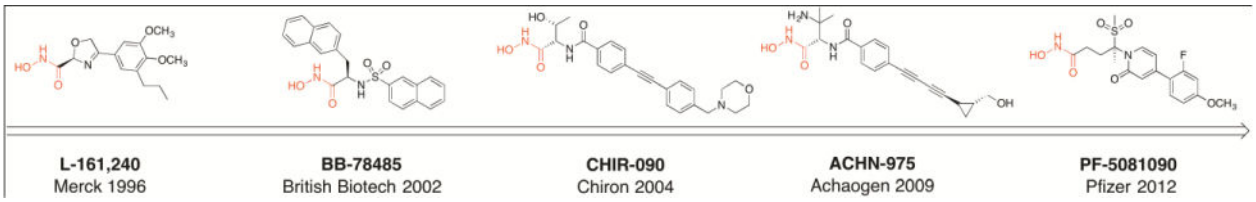

Figure 2.

Example of the prevalence of the hydroxamic acid MBP (highlighted in red) in a series of LpxC inhibitors over the course of 16 years of pharmaceutical research. Despite substantial changes in the overall molecular structure, the hydroxamic acid MBP remains unchanged across several independent drug discovery efforts.

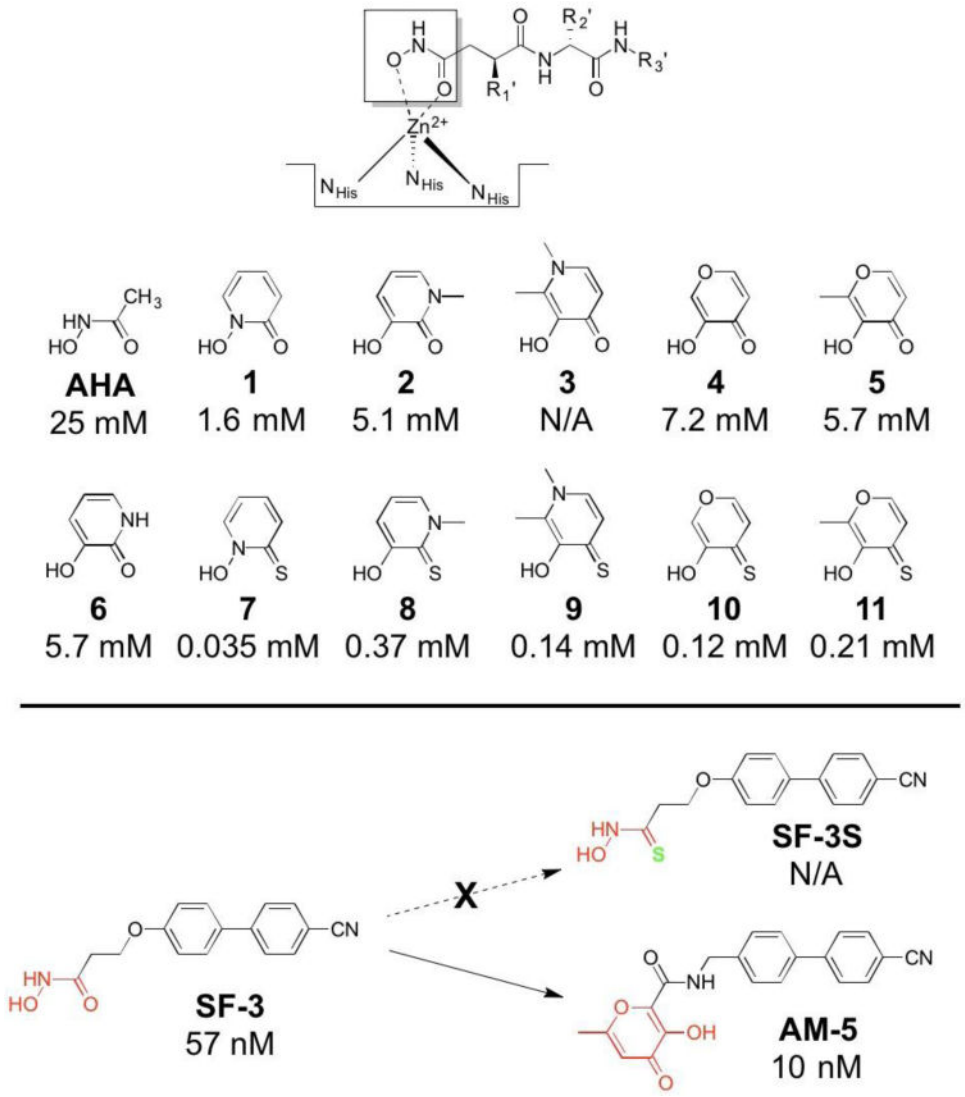

Figure 3.

Top: Scheme of a generic hydroxamic acid inhibitor binding to an MMP active site. Alternative MBPs (**1-11**) were all found to be more active than a hydroxamic acid MBP (acetohydroxamic acid, **AHA**). *Bottom*: To demonstrate the utility of alternative MBPs, a known MMP-3 inhibitor (**SF-3**) was modified (MBPs highlighted in red). Attempts to make a thiohydroxamic acid analog (**SF-3S**) were unsuccessful, but use of a maltol-based MBP (**AM-5**) led to a substantial improvement in activity. IC_{50} values shown below each compound are against MMP-3. Reproduced with permission from ref. [6]. Copyright 2004 American Chemical Society.

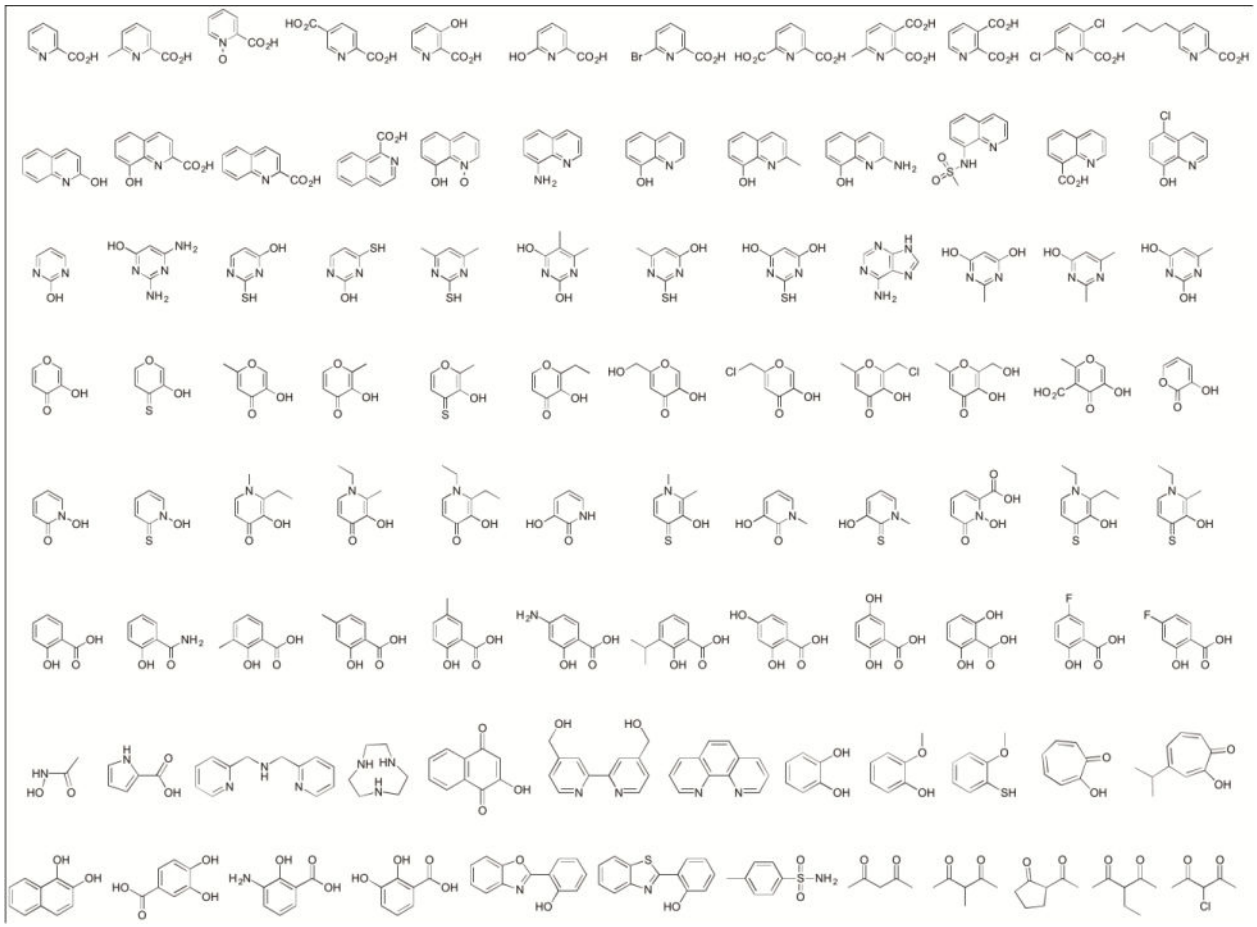

Figure 4.

A chelator fragment library (CFL) collection of MBPs specifically designed for FBDD against metalloenzyme targets. Reproduced with permission from ref. [20]. Copyright 2011 American Chemical Society.

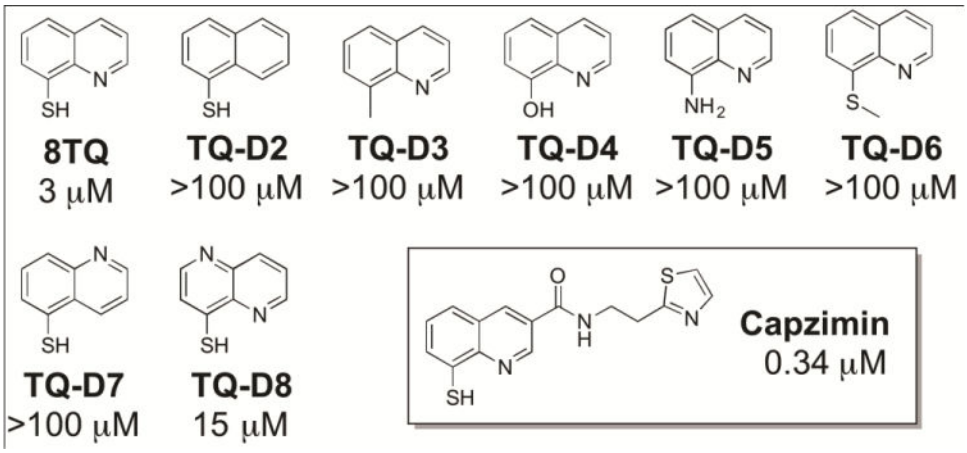

Figure 5.

FBDD leads to a first-in-class inhibitor of zinc-dependent Rpn11. The metal-binding motif of the hit fragment **8TQ** was confirmed by a series of derivatives that were inactive (**TQ-D2-7**). Only a derivative that maintains the ability to bind in a bidentate fashion with the preferred nitrogen/sulfur donor atom set (**TQ-D8**) retained activity. Fragment growth led to the lead compound 'capzimin'. IC_{50} values against Rpn11 are listed below each compound label.

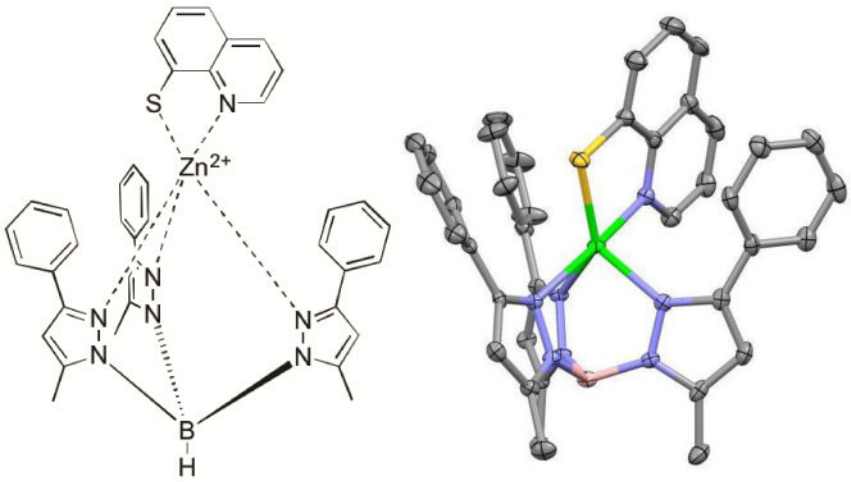

Figure 6.

Chemical illustration (*left*) and image of the X-ray structure (*right*) of [($Tp^{Me,Ph}$)Zn(**8TQ**)] showing a possible mode of **8TQ** binding to the active site Zn(II) ion in Rpn11. Thermal ellipsoids are shown at 50% probability. Hydrogen atoms are omitted for clarity. Color scheme: boron (pink), carbon (gray), nitrogen (blue), sulfur (yellow), and zinc (green). Reproduced with permission from ref [22]. Copyright 2017 American Chemical Society.

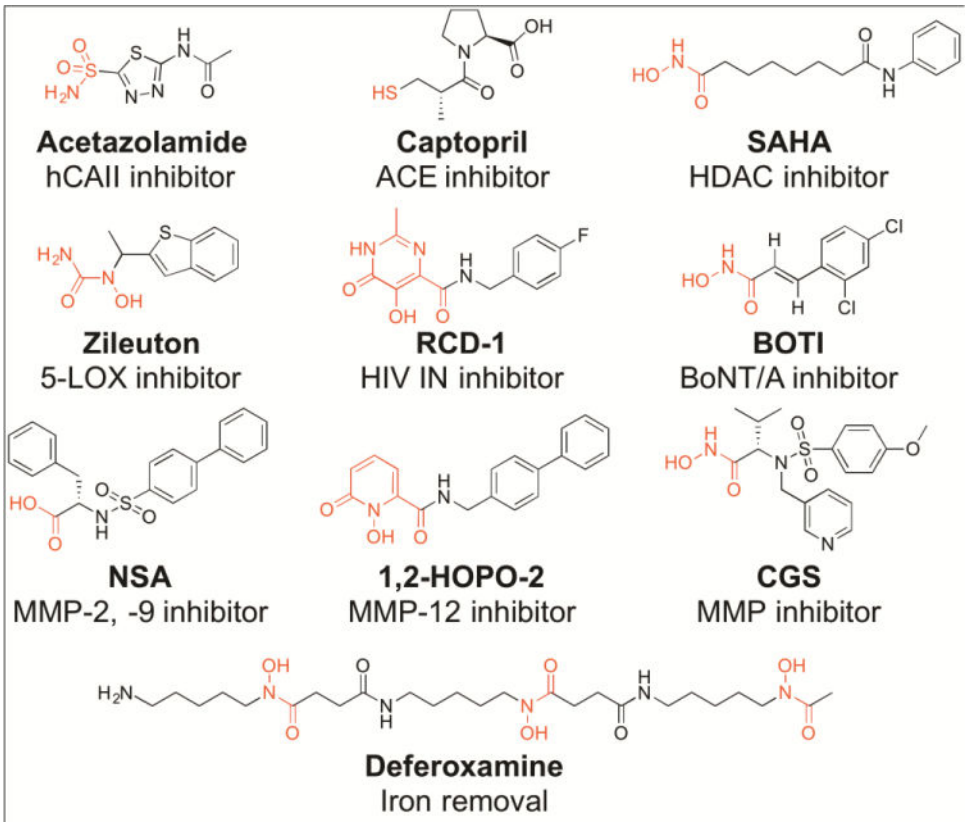

Figure 7.

Metalloenzyme inhibitors and their targets examined for their selectivity. MBPs are highlighted in red. Reproduced with permission from ref. [28]. Copyright 2013 American Chemical Society.

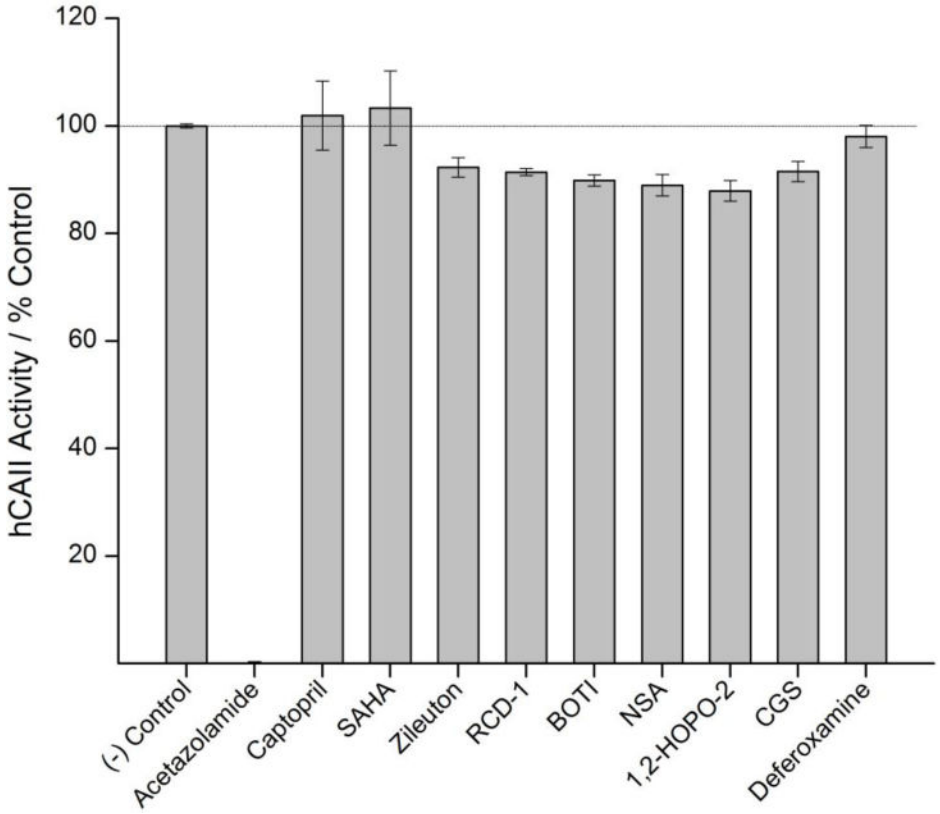

Figure 8.

Percent enzyme activity of hCAII in the presence of 10 μM of each metalloenzyme inhibitor. Reproduced with permission from ref. [28]. Copyright 2013 American Chemical Society.

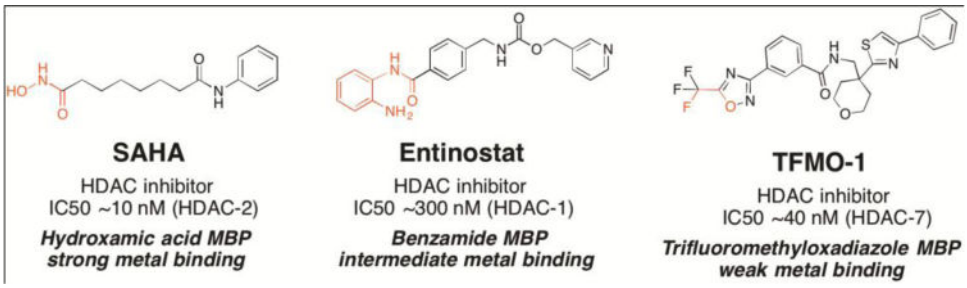

Figure 9.

HDAC inhibitors with different metal-binding ability (MBPs highlighted in red). IC_{50} values are listed for the HDAC isoform for which each inhibitor is most active. Reproduced with permission from ref. [30]. Copyright 2017 The Royal Society of Chemistry.