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Investigating the Selectivity of Metalloenzyme Inhibitors

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Abstract

The inhibitory activity of a broad group of known metalloenzyme inhibitors against a panel of metalloenzymes was evaluated. Clinically approved inhibitors were selected as well as several other reported metalloprotein inhibitors, in order to represent a broad range of metal binding groups (MBGs), including hydroxamic acid, carboxylate, hydroxypyridinonate, thiol, and *N*-hydroxyurea functional groups. A panel of metalloenzymes, including carbonic anhydrase (hCAII), several matrix metalloproteinases (MMPs), angiotensin converting enzyme (ACE), histone deacetylase (HDAC-2), and tyrosinase (TY) was selected based on their clinical importance for a range of pathologies. In addition, each inhibitor was evaluated for its ability to remove Fe³⁺ from holo-transferrin to gauge the ability of the inhibitors to access Fe³⁺ from a primary transport protein. The results show that the metalloenzyme inhibitors are quite selective for their intended targets, suggesting that despite their ability to bind metal ions, metalloprotein inhibitors are not prone to widespread off-target enzyme inhibition activity.

Introduction

Approximately one-third of proteins are metalloproteins, which serve to execute a wide array of functions in vivo, including regulating blood pH, facilitating matrix degradation, modulating DNA transcription, and many others.¹ Given the importance of these functions, metalloenzyme misregulation plays a significant role in human disease. Pathologies for which metalloenzymes are implicated include cancer,^{2, 3} heart disease,⁴ and HIV/AIDS.⁵ Given the impact of these diseases on human health, metalloenzyme inhibition offers an appealing approach to disease treatment. Indeed, sales of metalloprotein inhibitors account for billions of dollars in pharmaceutical sales annually.⁶

Typically, metalloenzyme inhibitors are drug-like small molecules that incorporate a metal binding group (MBG) in order to coordinate the active site metal ion. The MBG is appended to the drug-like portion of the inhibitor (often referred to as a 'backbone' group) via a linker. The backbone can take on many forms, reflective of the diversity in enzyme active sites. The metalloenzyme inhibitors in Figure 1 illustrate the variety of backbones, customized for their respective targets, which consist of assorted substituted aliphatic chains, aromatic rings, and heterocycles. In contrast to the diversity found in the inhibitor backbones, most metalloenzyme inhibitors incorporate a much narrower selection of MBGs. A search of the Protein Data Bank (PDB) for metalloprotein inhibitors show that the hydroxamic acid is the most common MBG, followed by carboxylic acids, thiols, and phosphonates. The use of only a few MBGs limits the chemical space being explored for the development of new

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Supporting Information **Available:** Inhibitor syntheses and characterization. This material is available free of charge via the Internet at <http://pubs.acs.org>.

therapeutics, particularly in light of the pharmacokinetic liabilities of inhibitors that contain hydroxamic acid^{3, 7-10} and thiol MBGs.^{11,12}

There appears to be a common opinion that inhibitors containing MBGs are more promiscuous than other small molecule compounds leading to off-target effects. These effects include indiscriminate metalloenzyme inhibition or metal ion dysregulation by metal ion removal from non-target metalloproteins, thus a perception of greater risk in obtaining clinically successful therapeutics.¹³⁻¹⁶ Although molecules that contain MBGs have the capacity to bind metal ions and metalloenzymes, to the best of our knowledge, a rigorous examination of the perceived liability of such compounds has not been reported in the scientific literature. Metal ion removal is an unlikely prospect based on existing data generated from the bioinorganic research community.^{17, 18} For example, transferrin, the major Fe³⁺ transport protein, which can exchange Fe³⁺ >100 times during its biologically useful lifetime,¹⁹ does not readily liberate its Fe³⁺ cargo, as it possesses an extremely high association constant (K_a) for Fe³⁺ (10^{22} M^{-1}).²⁰ Even at millimolar concentrations, iron-scavenging siderophores, such as enterobactin, require nearly an hour (in vitro) to remove iron from holo-transferrin.²¹

In order to test whether metalloenzyme inhibitors are more prone to inhibit off-target enzymes and/or remove metal ions from accessible sources, this report has tested the capacity of a select group of chelating inhibitors to inhibit a panel of metalloenzymes. In addition, the same inhibitors were examined for their activity against trypsin (a metal-independent protein) and for their ability to remove Fe³⁺ from the transport protein transferrin, which represents an important, exchangeable pool of transition metal ions in the bloodstream. By examining cross inhibition within a group of metalloenzymes, this study seeks to determine whether inhibitors of metalloenzymes are less specific than other small molecule enzyme inhibitors. The results presented here suggest that metalloenzyme inhibitors are not more promiscuous than other small molecule therapeutics. Furthermore, these inhibitors are incapable of removing Fe³⁺ from transferrin, even at concentrations far above any clinically relevant dose.

Inhibitor Screening – Target Selection

A group of ten metalloprotein inhibitors were selected for evaluation (Figure 1). The molecules were chosen to represent a diverse range of MBGs (7 distinct MBGs), target proteins (7 distinct targets), and therapeutic indications (at least 8 distinct pathologies). The inhibitors selected included both FDA-approved drugs as well as potent inhibitors from the scientific literature that were either purchased or synthesized according to published methods (Table 1).

Discovered in the 1930s,³³ carbonic anhydrase (CA) represents the oldest known class of Zn²⁺ metalloenzyme. The active site lies at the bottom of an open, cone-shaped pocket in the protein structure. The enzyme possesses a His₃ binding motif coordinated to the active site Zn²⁺ ion with a nucleophilic hydroxide occupying the fourth coordination site (Figure 2). Human CA (hCA) regulates blood pH, reversibly catalyzing the dehydration of bicarbonate to yield carbon dioxide (Figure 2). Systemic inhibition of hCA can have deleterious effects on the body in the form of acidosis as blood pH homeostasis is disrupted. Acetazolamide (diamox) is an FDA-approved drug whose clinical efficacy for treating glaucoma was first determined in 1954 by Becker.²² Acetazolamide inhibits many hCA isoforms (IC₅₀ ~25 nM) by binding the catalytic Zn²⁺ ion through a sulfonamide moiety (Figure 1). The drug disrupts the homeostasis between carbon dioxide and bicarbonate and as the concentration of bicarbonate decreases, water is drawn from the eye by osmosis, decreasing intraocular pressure, which proves to be an effective treatment for many cases of glaucoma.

Matrix metalloproteinases (MMPs) are a group of Zn^{2+} -dependent endopeptidases known for their potential to disrupt angiogenesis in malignant tumors and represent a prototypical metalloenzyme target in medicinal chemistry.³⁴⁻³⁶ MMP enzymes are capable of breaking down many forms of connective tissue, including gelatin (MMP-2 and -9) and elastin (MMP-12). The MMP active site contains a catalytic Zn^{2+} ion coordinated by a His_3 motif and a water molecule (Figure 2).³⁷ Three MMP inhibitors (Figure 1) were chosen for this study based on their different MBGs as well as known isoform selectivity. Developed by Tamura et al,²⁹ NSA (for *N*-sulfonylamino acid) is an MMP-2 and -9 selective inhibitor (IC_{50} 240 and 310 nM, respectively) that uses a carboxylic acid moiety as the MBG. A second MMP inhibitor, 1,2-HOPO-2, uses a hydroxypyridinone MBG, which is quite distinct from other MMP inhibitors. 1,2-HOPO-2 was prepared by Agrawal et al as an MMP-12 inhibitor, but shows a semi-selective profile with activity against other isoforms.³⁸ The third MMP inhibitor included in the study is the potent, broad spectrum compound CGS 27023A.³¹ Developed by Novartis Pharmaceuticals, CGS 27023A incorporates a hydroxamic acid MBG, the most common MBG for targeting MMPs and most other metalloenzymes. Despite the discovery of several potent broad-spectrum MMP inhibitors, no inhibitors have successfully completed clinical trials for any major disease indications (e.g. cancer, arthritis, etc.), due to problematic side effects, lack of efficacy in human trials, and other barriers.^{3, 7} Currently the only FDA-approved MMP inhibitor, doxycycline, is a treatment for periodontitis.³⁹

Angiotensin converting enzyme (ACE) is a Zn^{2+} dipeptidyl carboxypeptidase in which the catalytic Zn^{2+} ion is bound to the protein by a His_2Glu motif (Figure 2). ACE functions endogenously to generate the biologically active polypeptide angiotensin II from angiotensin I.⁴⁰ The cleaved product induces a vasoconstriction response that manifests as increased blood pressure. An important metalloenzyme inhibitor for treating hypertension, captopril (capoten, Squibb, patented in 1976),²³ incorporates a thiol group capable of coordinating the catalytic Zn^{2+} ion in the active site of ACE (Figure 1). Subsequent ACE inhibitors moved away from the thiol MBG, in order to avoid the deleterious clinical effects associated with some thiols. Second-generation ACE inhibitors generally utilized carboxylates as MBGs, requiring further backbone enhancements to achieve comparable levels of inhibition to captopril.⁴¹ Due to the similarity in function and active site between MMPs and ACE, ACE inhibitors have been previously studied and observed to cross-inhibit MMPs.⁴² Captopril has also been observed to inhibit mushroom tyrosinase, another metalloprotein examined in this study (see below).⁴³

The histone deacetylase (HDAC) family of Zn^{2+} metalloenzymes, in conjunction with histone acetyltransferases (HAT), regulate gene expression. HDACs can be segregated into Zn^{2+} -dependent metalloenzymes (Classes I, IIa, IIb, and IV) and the metal-free sirtuins (Class III). For this study, only the Zn^{2+} metalloenzymes were considered. Deacetylation results in the positively charged ϵ -amines of lysine interacting strongly with the negatively charged phosphate backbone of DNA, serving to coil DNA tighter onto the histone scaffold comprising chromatin. At the site of increased DNA constriction translation is turned off and genes are repressed. The genes down-regulated by HDACs include those coding for cancer cell apoptosis, supplying the impetus for inhibitor development.^{44, 45} The HDAC family incorporates an active site consisting of a Zn^{2+} ion bound by a HisAsp_2 motif (Figure 2). Inhibitors of HDACs, such as suberoylanilide hydroxamic acid (SAHA, vorinostat, Merck), activate tumor suppression genes within cancerous cells leading to growth arrest and cell death.⁴⁶ SAHA, a first-in-class, broad spectrum HDAC inhibitor, is a relatively simple molecule incorporating a hydroxamic acid MBG, an aliphatic linker, and an aromatic capping group (Figure 1). SAHA was discovered in 1996 by Richon et al²⁵ and approved by the FDA as a treatment for cutaneous T-cell lymphoma in 2006.⁴⁷ Currently, there is only

one other HDAC inhibitor that has received FDA approval, romidepsin, also for treating cutaneous T cell lymphoma.⁴⁸

Mushroom tyrosinase (TY) is a dinuclear Cu^{2+} oxidase controlling the production of melanin. The two active site Cu^{2+} ions are bound by six histidine residues (three to each metal ion, Figure 2) and reversibly bind either a bridging μ -oxide or peroxide species.⁴⁹ TY converts tyrosine to L-DOPA and subsequently L-DOPA to dopaquinone. Dopaquinone then proceeds through a series of intermediates, eventually polymerizing to generate melanin.⁴⁹ Inhibition of TY decreases melanin production and subsequently results in skin whitening. Consequently, TY inhibitors are of interest to the cosmetics industry.⁵⁰ The natural product tropolone was used as an inhibitor of TY for this study (IC_{50} ~400 nM); notably, a crystal structure of tropolone bound to TY shows the molecule occupying the substrate channel of the enzyme without coordinating the active site metal ions (PDB: 2Y9X).⁵¹ However, the tropolone MBG is known to inhibit several other metalloenzymes⁴⁹ and has been used has been used successfully as a lead for inhibitor development.⁵²

Finally, transferrin, the primary iron transport protein, was included in this study to represent metal transport and homeostasis proteins. Transferrin serves a dual purpose, transporting iron throughout the body while scavenging any free or loosely bound iron in the serum. There are two Fe^{3+} ion binding sites in transferrin, an N-terminal domain and a C-terminal domain, with both sites possessing a high affinity for Fe^{3+} ions.⁵³ Transferrin is extremely effective at binding Fe^{3+} ions, possessing an association constant of 10^{22} M^{-1} .²⁰ As a result, there is essentially no free iron in the serum of healthy humans. Pathogens have developed potent Fe^{3+} chelators known as siderophores in order to obtain the necessary Fe^{3+} ions for proliferation.⁵⁴ The aim of the transferrin assays was to determine if any of the metalloenzyme inhibitors studied possess the capacity to remove Fe^{3+} ions from holo-transferrin. Deferoxamine (desferal) is a siderophore originally isolated from *Streptomyces pilosus* by Bickel et al in 1960.³² Deferoxamine (Figure 1) is a clinically approved therapeutic for iron overload and was chosen for screening against the panel of metalloenzymes in this study. Despite the efficacy of deferoxamine for eliminating iron, it does not remove Fe^{3+} ions from holo-transferrin on a rapid timescale⁵⁵ (although many factors such as ionic strength, pH, and others can influence removal kinetics).^{56, 57} Deferiprone (L1, Figure 1), another clinically approved Fe^{3+} chelating agent was also included as a positive control, as it is known to rapidly remove Fe^{3+} ions from holo-transferrin.²¹

Other Inhibitors Tested

Human immunodeficiency virus integrase (HIV-1 IN) incorporates viral DNA into the host DNA, generating reactive CpA 3'-hydroxyl ends on the viral cDNA and subsequently fusing the viral DNA into the host genome in a process known as strand transfer. The two Mg^{2+} ions in the dinuclear active site are responsible for activating the DNA primer 3'-hydroxyl group.⁶³ Raltegravir is a first-in-class inhibitor of HIV-1 IN developed by Merck that received FDA approval in 2007 (Figure 1).⁶⁴ Inhibitors based on the MBGs similar to that of raltegravir were developed by Agrawal et al to elucidate the effect of the MBG on HIV integrase inhibition efficacy.²⁷ One compound used in the latter study, RCD-1, possesses the same 5-hydroxy-3-methylpyrimidin-4(3H)-one (HMPO) MBG found in raltegravir, and shows effective strand transfer inhibition. RCD-1 (IC_{50} ~60 nM) was chosen as representative of raltegravir, because it is more synthetically accessible and shares a common MBG and backbone with the FDA approved drug.

An extremely potent family of toxins, botulinum neurotoxins (BoNTs) are the Zn^{2+} -dependent metalloenzyme toxins produced by *Clostridium botulinum*. Due to the paucity of available treatments for botulism, synthetic efforts have been directed towards developing a

potent inhibitor of botulinum neurotoxin. The BoNT inhibitor selected for this study was designed by the Janda group²⁸ (BOTI, Figure 1) around the ubiquitous hydroxamic acid MBG with a backbone to impart potency against BoNT serotype A (BoNT/A) with an IC₅₀ of 410 nM.

5-Lipoxygenase (5-LOX) is a non-heme Fe³⁺-dependent dioxygenase responsible for smooth muscle contractions observed in asthma and allergic reactions.⁶⁵ 5-LOX functions endogenously to convert *cis*-polyunsaturated fatty acids into leukotrienes, first adding molecular oxygen to the fifth carbon on the fatty acid, generating a hydroperoxide, and subsequently dehydrating the hydroperoxide to yield an epoxide-containing leukotriene.⁶⁶ The leukotrienes trigger an inflammatory response leading to bronchoconstriction. Correspondingly, inhibitors of 5-LOX activity are sought for their therapeutic applications towards treating asthma. One such inhibitor, zileuton (zyflo), is an FDA-approved drug for the prophylactic treatment of asthma (IC₅₀ 410 nM).²⁶ Zileuton was developed by Abbot Laboratories in 1991²⁶ and was approved for distribution in 1996.

Results

Inhibition of MMPs

The activity of MMP-2 and -12 were monitored via a kinetic assay that measures the increase in fluorescence upon cleavage of a peptidic substrate (OmniMMP).⁶⁷ The assay was performed in buffer (50 mM HEPES, 10 mM CaCl₂, 0.05% Brij-35, pH 7.5) in which the substrate was added to a mixture of protein and inhibitor which had been preincubated at 37 °C for 30 min. The effect of all ten inhibitors against these proteinases is compared in Figure 3. At 10 μM, CGS exhibited greater than 95% inhibition of both MMPs. Although NSA is reported to be an MMP-2 and -9 isoform inhibitor (IC₅₀ 240 and 310 nM, respectively) at high concentrations, such as 10 μM used here, isoform selectivity was abolished, resulting in total inhibition of MMP-2 and -12. The third MMP inhibitor, 1,2-HOPO-2, retained some selectivity towards MMP-12, achieving 100% inhibition against MMP-12, but only 80% inhibition against MMP-2. At a concentration of 10 μM, the other hydroxamic acid-based inhibitors in the study, SAHA and BOTI, were observed to decrease MMP activity by <25%. RCD-1 also decreased MMP-2 activity by ~50%, but this is not entirely surprising based on overall structural similarities (MBG, linker, and backbone) to 1,2-HOPO-2. The remaining compounds demonstrated little activity against MMP-2 and -12 (Figure 3). It is particularly interesting to note that despite previous reports in which captopril was observed to inhibit MMP-2 in cell studies,⁴² less than 10% inhibition was observed in our experiments.

Inhibition of hCAII

The activity of hCAII was evaluated using a common procedure by monitoring the esterase activity of the enzyme with *p*-nitrophenyl acetate as a substrate.⁶⁸ The assay was performed in buffer (50 mM Tris-SO₄, pH 8.0) in which the substrate was added to a mixture of protein and inhibitor which had been preincubated at 30 °C for 10 min. Upon cleavage of the acetate group, *p*-nitrophenolate anion absorbs at 405 nm allowing for observation of hCAII activity as a function of increasing absorbance over time. The effects of the inhibitors against hCAII are compared in Figure 4. Of the inhibitors screened, only the on-target inhibitor, acetazolamide, significantly inhibited hCAII at a concentration of 10 μM, resulting in essentially complete (100%) inhibition. All the remaining inhibitors were inactive against hCAII. Despite the fact that seven of the ten inhibitors in this study are designed for Zn²⁺ metalloenzymes, and hCAII has a relatively open active site (Figure 2), only minor cross-inhibition was observed. These results suggest that even potent MBGs for Zn²⁺ ions such as

hydroxamic acids and thiols will not indiscriminately inhibit Zn^{2+} metalloenzymes in the absence of favorable inhibitor backbone-protein interactions.

Inhibition of HDAC-2

The activity of HDAC-2 was measured following the deacetylation of a small, dye-containing peptide. The assay was performed using a commercially available kit according to manufacturer instructions. The assay was performed in buffer (supplied by the manufacturer) in which the substrate was added to a mixture of protein and inhibitor that had been preincubated at room temperature for 5 min. The natural product trichostatin A (TSA) is utilized as part of the endpoint assay to quench enzyme activity and was also used as an added positive control in this study. At 10 μM , SAHA and TSA completely prevent substrate deacetylation (Figure 5). BOTI also demonstrated some capacity for inhibiting HDAC, presumably due to the structural similarities between the two inhibitors. BOTI is a relatively simple hydroxamic acid, possessing a MBG, linker, and capping group typically seen in HDAC inhibitor design.⁴⁵ Interestingly, the third hydroxamic acid, CGS, did not demonstrate any inhibitory activity against HDAC-2. The active site in HDAC-2 is accessed through a narrow pore in the protein, typically filled by the linker of an HDAC inhibitor that presumably cannot accommodate the steric bulk of CGS. No inhibition was observed among the remaining inhibitors.

Inhibition of ACE

ACE activity was determined as previously reported using the OmniMMP fluorogenic substrate.⁶⁹ In addition to MMPs, OmniMMP has been determined to be a suitable substrate for a variety of Zn^{2+} -dependent metalloproteases.⁶⁹ The assay was performed in buffer (50 mM MES, pH 6.5) in which the substrate was added to a mixture of protein and inhibitor which had been preincubated at 37 °C for 10 min. The FDA-approved ACE inhibitor captopril served as the positive control in this assay. Two other inhibitors from this study were observed to inhibit at 10 μM , 1,2-HOPO-2 and SAHA (Figure 6). Inhibition by 1,2-HOPO-2 is not particularly surprising based on the degree of substrate overlap between MMPs and ACE (i.e. the fluorogenic OmniMMP substrate is used in both assays).⁶⁹ Substrate overlap also explains why CGS, a potent, broad spectrum MMP inhibitor, also showed weak inhibition of ACE; however, it is interesting to note that the MMP inhibitor NSA did not inhibit ACE, showing that high selectivity between MMPs and ACE inhibitors can be achieved.

Inhibition of TY

TY catalyzes two sequential reactions, oxidation of tyrosine to L-DOPA (L-3,4-dihydroxyphenylalanine) and oxidation of L-DOPA to L-dopaquinone.⁶⁷ The product, L-dopaquinone, polymerizes to generate melanin, which can be observed spectrophotometrically by an increase in absorbance at 475 nm.⁷⁰ In this study, L-DOPA was used as the substrate for assaying TY activity. The assay was performed in buffer (10 mM NaH_2PO_4 , pH 6.8) in which the substrate was added to a mixture of protein and inhibitor that had been preincubated at room temperature for 10 min. The reaction was allowed to proceed for 10 min before recording the change in absorbance. Interestingly, none of the inhibitors tested were found to reduce TY activity at 10 μM (Figure 7). Only the positive control, tropolone (IC_{50} ~400 nM), was effective at shutting down TY activity. This is somewhat surprising, as studies on a simple fragment library showed that TY is readily inhibited by a wide range of MBGs.⁶⁷ Even RCD-1, which is a dinuclear metalloenzyme inhibitor, did not inhibit TY. These findings reinforce the notion that enzymatic selectivity is imparted to a metalloenzyme inhibitor by both the MBG and backbone constituents, as was recently demonstrated by Fullagar et al⁵² in which a tropolone derivative designed as an

inhibitor of the Zn^{2+} -dependent metalloenzyme LasB simultaneously lost efficacy for TY inhibition.

Inhibition of Trypsin

Trypsin is a serine endopeptidase (metal-independent enzyme) that participates in the degradation of proteins in the digestive system. We sought to include the possible cross-reactivity of the metalloprotein inhibitors on one, common metal-independent enzyme. Trypsin was an ideal choice because of its availability, facile assay, and similar function (proteinase) to many of the other enzymes (e.g. MMPs) examined in this study. The assay was performed using a commercially available kit according to manufacturer instructions. The assay was performed in buffer (supplied by the manufacturer) in which the substrate was added to a mixture of protein and inhibitor that had been preincubated at room temperature for 10 min. An irreversible inhibitor of trypsin, tosyllysine chloromethyl ketone hydrochloride (TLCK), was included as a positive control. None of the metalloprotein inhibitors showed any inhibition of trypsin at a concentration of 10 μM (Figure 8). Notably, trypsin is an integral part of the HDAC-2 activity assay used in this study, confirming that inhibitor activity against HDAC-2 is not due to inhibition of trypsin (Figure 5).

Transferrin Fe^{3+} Removal Assay

Biological access to transition metal ions in vivo is highly regulated and restricted. To explore whether the potent inhibitors in Figure 1 could access biological sources of transition metal ions, their ability to extract Fe^{3+} from the serum metalloprotein transferrin was examined. The capacity of an inhibitor to remove Fe^{3+} from transferrin was determined by using standard assay conditions,^{57, 71, 72} where the change in absorbance at 466 nm of a 100 μM solution (50 mM Tris, pH 7.4, 150 mM NaCl, 20 mM NaHCO_3) of Fe^{3+} -saturated transferrin (holo-transferrin) is monitored over the course of 1 hour in the presence of an inhibitor. The only inhibitor observed to remove Fe^{3+} from transferrin was 1,2-HOPO-2 (Table 2), which is consistent with observations that HOPO-based ligands are known to effect iron removal from transferrin. Indeed, the positive control for the metal removal assays, deferiprone, is an *N*-methylated 3,4-HOPO derivative. Deferoxamine is known to remove Fe^{3+} ions from ferritin, but not transferrin, so it is not surprising that deferoxamine remained innocuous in this assay. No data was obtained for RCD-1, as it interfered with the assay conditions.

Discussion

As more metalloproteins are identified and validated as clinical targets, the potential for cross-inhibition by inhibitors will become an increasing concern. Given the prevalence of metal ion cofactors in biology, the possibility for indiscriminate metal binding could have a range of deleterious effects. Inhibitors bound to unintended metalloproteins decreases the bioavailability of inhibitor for the target enzyme, as well as potentially causing undesired off-target effects. The liberation of loosely coordinated metal ions could also lead to Fenton chemistry, resulting in damaged biomolecules and possible loss-of-function. To the best of our knowledge, no study has been performed to investigate the degree of interaction between a metal chelating, metalloprotein inhibitor and a broad range of metalloproteins. A prior study investigated small (<175 amu) metal binding fragments (i.e. MBGs) against a similar panel of metalloenzymes and found that these fragments showed a range of inhibitory behavior, from broad-spectrum to highly selective.⁶⁷

The hydroxamic acid MBG is the prototypical chelator for metalloenzyme inhibitor design, and consequently three hydroxamate-based compounds were included in this study (Figure 1). The hydroxamic acid CGS is an extremely potent, broad-spectrum MMP inhibitor, and

yet shows little off-target metalloenzyme inhibition (Figure 9). In contrast, the other hydroxamates, SAHA and BOTI, both showed some limited off-target inhibition. The selectivity displayed by these hydroxamate inhibitors presumably stems from the composition of the backbone group. The backbone of CGS is complex and has been finely tuned to fit into the active site of MMPs, imparting both potency and selectivity to the target. In contrast, SAHA and BOTI are much simpler inhibitors that possess backbones designed to fill a narrow channel in the enzymes, with little specificity built in beyond the linker length between the MBG and the capping group. Although SAHA and BOTI do exhibit off-target inhibition, it is still important to note that the degree of inhibition is weak. Perhaps the same factors that result in their lower specificity (i.e. their simplicity), also restricts these inhibitors to being weak off-target inhibitors, hence incapable of matching the activity of on-target inhibitors, and not exhibiting problematic cross-reactivity at therapeutically-relevant concentrations.

Three different MMP inhibitors, NSA, CGS, and 1,2-HOPO-2, include three different MBGs: a carboxylic acid, hydroxamic acid, and a hydroxypyridinone, respectively (Figure 1). These inhibitors were selective for MMPs with the exception of 1,2-HOPO-2, which was observed to inhibit ACE, an enzyme with great similarity to the MMPs. This off-target inhibition might be attributed to the more tightly coordinating MBG found in 1,2-HOPO-2 versus NSA or CGS.⁶⁷ Indeed, CGS and NSA are highly active MMP inhibitors, but neither showed activity against ACE (Figure 9). Hence, the importance of the MBG to inhibition is best considered within the context of the full structure of the inhibitor when optimizing potency and selectivity.

Although still quite low, the predominant off-target inhibition is observed in the cases of RCD-1, BOTI, and 1,2-HOPO-2, all of which are not FDA approved compounds and thereby share a degree of simplicity relative to approved compounds. RCD-1 is a substantially truncated version of raltegravir, BOTI is a small compound that bears a strong resemblance to simple HDAC inhibitors, and 1,2-HOPO-2 relies on a simple biphenyl moiety as the backbone. Also, all three compounds possess different MBGs, precluding the possibility that an MBG alone will impart promiscuity. Based on the high concentrations of compounds used in these experiments, it is clear that none of these inhibitors exhibit significant off-target inhibition of the enzymes tested.

In an effort to probe the most extreme form of metalloprotein and metal ion homeostasis disruption, Fe³⁺ stripping studies from holo-transferrin were performed. No inhibitor, with the exception of 1,2-HOPO-2, could remove iron from transferrin (Table 2). It is also important to stress that millimolar concentrations of 1,2-HOPO-2 were required to observe iron removal on a reasonable timescale. Given that all of the metalloenzyme inhibitors studied are nanomolar inhibitors of their respective targets, and, at most, reach peak serum concentrations in the low micromolar range,⁷³⁻⁷⁷ the possibility of metal stripping from transferrin is highly unlikely. Advancements in bioinorganic chemistry indicate that metal ion homeostasis is tightly regulated and that intracellular concentrations of free transition metal ions are very low, further suggesting that metalloprotein inhibitors are unlikely to perturb metal trafficking.^{78, 79}

Conclusion

A variety of known, potent metalloenzyme inhibitors were screened against a representative panel of metalloproteins. The results indicate that at very high concentrations of inhibitor (10 μ M), off-target inhibition is limited, even when inhibitors contain similar or identical MBGs. Not surprisingly, the limited off-target inhibition observed is greatest among metalloenzymes incorporating the same catalytic metal, active site structure, and

functionality. In addition, off-target inhibition was more common among early-stage lead compounds (e.g. 1,2-HOPO-2, BOTI) for which complete drug development efforts have not been applied. Almost all FDA-approved, full-length inhibitors showed no off-target inhibition, regardless of the MBG, suggesting that inhibitor selectivity is a combination of the MBG and the backbone. Therefore, the findings presented here strongly suggest that metal chelating inhibitors of metalloproteins present no particular risk for indiscriminate off-target inhibition of metalloenzymes as a result of non-specific metal chelation. Therefore, it appears that the development of metalloprotein inhibitors present no more risk for non-specific activity than small molecule inhibitors of metal-independent enzymes. These results should help to spark renewed interest in such targets, where a wealth of therapeutic possibilities await.^{14, 80}

Experimental

Materials

All reagents and acetazolamide, captopril, zileuton, and deferoxamine were obtained from Sigma-Aldrich. RCD-1, 1,2-HOPO-2, BOTI, CGS, SAHA, and TSA were prepared by literature methods.^{24, 27-31} The purity of all synthesized compounds was determined to be 95% by analytical HPLC. UV/Visible spectra were recorded with a Perkin-Elmer Lambda 25 spectrophotometer. Absorbance assays were performed using a BioTek ELx800 microplate reader. Fluorescence assays were performed using either a BioTek FLx800 microplate reader or a BioTek Synergy HT microplate reader.

Human Carbonic Anhydrase II Activity Assays⁶⁸

hCAII was expressed and purified as previously described.⁸¹ Assays were carried out in clear Costar 96-well plates. Each well contained a volume of 100 μ L including buffer (50 mM Tris-SO₄, pH = 8.0), hCAII (200 nM), inhibitor (100 μ M), and *p*-nitrophenyl acetate (500 μ M). The enzyme and inhibitor were incubated in solution at 30 °C for 10 min prior to the addition of the substrate to initiate the reaction. The change in absorbance was monitored for 20 min at 405 nm. The negative control wells, containing no inhibitor, were arbitrarily set as 100% activity. Readings from background wells, which did not contain protein, were subtracted from the active assay wells in order to account for background hydrolysis activity caused by the buffer. The assays were performed in triplicate, and each assay contained each inhibitor in triplicate. The data were normalized to values measured for uninhibited enzyme. Assays were reported as mean $\pm$ standard deviation.

MMP Assay³⁰

MMP-2 and -12, and OmniMMPTM fluorogenic substrate were purchased from Enzo Life Sciences (Farmingdale, NY). The assays were carried out in white NUNC 96-well plates. Each well contained a volume of 100 μ L including buffer (50 mM HEPES, 10 mM CaCl₂, 0.05% Brij-35, pH 7.5), human recombinant MMP (1.16 U of MMP-2, 0.007 U of MMP-12, ENZO Life Sciences), inhibitor (100 μ M), and fluorogenic OmniMMP substrate (4 μ M Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂•AcOH, ENZO Life Sciences). The enzyme and inhibitor were incubated in solution at 37 °C for 30 min, followed by the addition of the substrate to initiate the reaction. The change in fluorescence was monitored for 30 minutes on a BioTek FLx 800 fluorescence plate reader with excitation and emission wavelengths at 320 and 400 nm, respectively. The negative control wells, containing no inhibitor, were arbitrarily set as 100% activity. MMP activity was defined as the ratio of fluorescence increase in the inhibitor wells relative to the negative control wells, expressed as a percentage. The assays were performed in triplicate, and each assay contained each inhibitor in triplicate. The data were normalized to values measured for uninhibited enzyme. Assays were reported as mean $\pm$ standard deviation.

HDAC Assay

The HDAC assay kit (green) was purchased from BPS Bioscience (San Diego, CA). The assays were carried out in black, low binding NUNC 96-well plates. Each well contained a volume of 50 μ L including buffer (BPS Bioscience, Catalog No. 50031), HDAC-2 (30 ng, BPS Bioscience, Catalog No. 50002), inhibitor (10 μ M), BSA (1 mg/mL, Sigma-Aldrich), and HDAC substrate 2 (20 μ M, BPS Bioscience, Catalog No. 50038). Prior to adding substrate, the plate was preincubated for 5 min. Upon addition of substrate the plate was incubated at 37 °C for 30 min. HDAC assay developer (50 μ L, BPS Bioscience, Catalog No. 50030) was added to each well and the plate incubated for 15 min at room temperature. The fluorescence was recorded at excitation and emission wavelengths of 485 and 528 nm, respectively. Blank wells containing no inhibitor or protein were subtracted from all wells. The assays were performed in triplicate, and each assay contained each inhibitor in triplicate. The data were normalized to values measured for uninhibited enzyme. Assays were reported as mean $\pm$ standard deviation.

ACE Assay⁶⁹

ACE was purchased from R&D Systems (Minneapolis, MN). The assays were carried out in white NUNC 96-well plates. Each well contained 100 μ L including buffer (50 mM MES, pH 6.5), human recombinant ACE/CD143 somatic form (20 ng, R&D Systems), inhibitor (10 μ M), and fluorogenic OmniMMP substrate (4 μ M Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂•AcOH, ENZO Life Sciences). The enzyme and inhibitor were incubated in solution at 37 °C for 10 min prior to substrate addition. The change in fluorescence was monitored for 30 min with excitation and emission wavelengths at 320 and 400 nm, respectively. The assays were performed in triplicate, and each assay contained each inhibitor in triplicate. The data were normalized to values measured for uninhibited enzyme. Assays were reported as mean $\pm$ standard deviation.

Tyrosinase Assay⁷⁰

The assays were carried out in clear Costar 96-well plates. Each well contained a volume of 100 μ L including buffer (10 mM NaH₂PO₄, pH=6.8), mushroom tyrosinase (30 U, Sigma-Aldrich), inhibitor (10 μ M), and L-DOPA (0.5 mM). The enzyme and inhibitor were incubated in solution at room temperature for 10 min. Longer incubation times (up to 30 min) did not significantly change the assay results. A background absorbance was recorded at 475 nm using a BioTek ELx 808 colorimetric plate reader. L-DOPA was added to each well concurrently to initiate the reaction. After 10 min a second absorbance reading was recorded at 475 nm. The background absorbance was subtracted from the second absorbance. The negative control wells, containing no inhibitor, were arbitrarily set as 100% activity. The assays were performed in triplicate, and each assay contained each inhibitor in triplicate. The data were normalized to values measured for uninhibited enzyme. Assays were reported as mean $\pm$ standard deviation.

Trypsin Assay

The trypsin assay kit was purchased from BioVision (San Francisco, CA). The assays were performed using a commercially available colorimetric assay kit purchased from BioVision. The assays were carried out in clear Costar 96-well plates. Each well contained 100 μ L including buffer (BioVision, Catalog No. K771-100-1), trypsin (100 mU, TPCK treated, Worthington Biochemical Corporation), inhibitor (10 μ M), and substrate (BioVision, Catalog No. K771-100-2). The enzyme and inhibitor were incubated in solution at 25 °C for 10 min prior to substrate addition. The change in absorbance was monitored for 30 min at 400 nm. The assays were performed in triplicate, and each assay contained each inhibitor in

triplicate. The data were normalized to values measured for uninhibited enzyme. Assays were reported as mean $\pm$ standard deviation.

Kinetic Studies of Iron Removal from Transferrin^{57,71,72}

A solution of human holo-transferrin (100 μ M, Sigma-Aldrich) in buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 20 mM NaHCO₃) incubated at 37 °C for 5 min. Inhibitor was added to the solution (0.1-3 mM) at a final volume of 600 μ L. Immediately following addition of the inhibitor, the solution was monitored at 466 nm every 20 s for 30-60 min using a PerkinElmer Lambda 25 UV/VIS Spectrometer equipped with a thermostat jacketed cell maintained at 37 °C using 1 cm quartz cuvettes. Experiments were performed in triplicate at each inhibitor concentration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Holm RH, Kennepohl P, Solomon EI. Structural and Functional Aspects of Metal Sites in Biology. *Chem Rev.* 1996; 96:2239–2314. [PubMed: 11848828]
2. Ma X, Ezzeldin HH, Diasio RB. Histone deacetylase inhibitors: current status and overview of recent clinical trials. *Drugs.* 2009; 69:1911–1934. [PubMed: 19747008]
3. Fisher JF, Mobashery S. Recent advances in MMP inhibitor design. *Cancer Metastasis Rev.* 2006; 25:115–136. [PubMed: 16680577]
4. Dive V, Chang CF, Yiotakis A, Sturrock ED. Inhibition of zinc metalloproteinases in cardiovascular disease--from unity to trinity, or duality? *Curr Pharm Des.* 2009; 15:3606–3621. [PubMed: 19925415]
5. Dubey S, Satyanarayana YD, Lavania H. Development of integrase inhibitors for treatment of AIDS: An overview. *Eur J Med Chem.* 2007; 42:1159–1168. [PubMed: 17367896]
6. Raeside, A.; Strickland, I. *World Preview 2018: Embracing the Patent Cliff.* EvaluatePharma; London: 2012.
7. Coussens LM, Fingleton B, Matrisian LM. Matrix Metalloproteinase Inhibitors and Cancer—Trials and Tribulations. *Science.* 2002; 295:2387–2392. [PubMed: 11923519]
8. Frey RR, Wada CK, Garland RB, Curtin ML, Michaelides MR, Li J, Pease LJ, Glaser KB, Marcotte PA, Bouska JJ, Murphy SS, Davidsen SK. Trifluoromethyl ketones as inhibitors of histone deacetylase. *Bioorg Med Chem Lett.* 2002; 12:3443–3447. [PubMed: 12419380]
9. Reiter LA, Robinson RP, McClure KF, Jones CS, Reese MR, Mitchell PG, Otterness IG, Bliven ML, Liras J, Cortina SR, Donahue KM, Eskra JD, Griffiths RJ, Lame ME, Lopez-Anaya A, Martinelli GJ, McGahee SM, Yocum SA, Lopresti-Morrow LL, Tobiasen LM, Vaughn-Bowser ML. Pyran-containing sulfonamide hydroxamic acids: potent MMP inhibitors that spare MMP-1. *Bioorg Med Chem Lett.* 2004; 14:3389–3395. [PubMed: 15177439]
10. Kantharaj, E.; Jayaraman, R. Histone deacetylase inhibitors as therapeutic agents for cancer therapy: Drug metabolism and pharmacokinetic properties. In: Rundfeldt, C., editor. *In Drug Development - A Case Study Based Insight into Modern Strategies.* InTech; 2011. p. 101-120.
11. Lin JH, Lu AYH. Role of pharmacokinetics and metabolism in drug discovery and development. *Pharmacol Rev.* 1997; 49:403–449. [PubMed: 9443165]

12. Edmonds S, Gibb A, Sim E. Effect of thiol compounds on human complement component C4. *Biochem J.* 1993; 289:801–805. [PubMed: 8435078]
13. Campion C, Davidson AH, Dickens JP, Crimmin MJ. Hydroxamino acid based collagenase inhibitors. 1994 US 5310763.
14. Schotzinger RJ, Hoekstra WJ. Metalloenzyme inhibitors using metal binding moieties in combination with targeting moieties. 2011 US 2011/0015158 A1.
15. Rishton GM. Nonleadlikeness and leadlikeness in biochemical screening. *Drug Discovery Today.* 2003; 8:86–96. [PubMed: 12565011]
16. Vickers CJ, Olsen CA, Leman LJ, Ghadiri MR. Discovery of HDAC Inhibitors That Lack an Active Site Zn²⁺-Binding Functional Group. *ACS Med Chem Lett.* 2012; 3:505–508.
17. Krezel A, Maret W. Thionein/metallothionein control Zn(II) availability and the activity of enzymes. *J Biol Inorg Chem.* 2008; 13:401–409. [PubMed: 18074158]
18. Kiefer LL, Krebs JF, Paterno SA, Fierke CA. Engineering a cysteine ligand into the zinc binding site of human carbonic anhydrase II. *Biochemistry.* 1993; 32:9896–9900. [PubMed: 8399158]
19. Harford, J.; Rouault, T.; Heubers, H.; Klausner, R. *The Molecular Basis of Blood Diseases.* 2. W B Saunders Co; Philadelphia: 1994. p. 351-378.
20. Aisen P, Listowsky I. Iron transport and storage proteins. *Annu Rev Biochem.* 1980; 49:357–393. [PubMed: 6996567]
21. Hamilton D, Turcot I, Stintzi A, Raymond K. Large cooperativity in the removal of iron from transferrin at physiological temperature and chloride ion concentration. *J Biol Inorg Chem.* 2004; 9:936–944. [PubMed: 15517438]
22. Becker B. Decrease in intraocular pressure in man by a carbonic anhydrase inhibitor, Diamox: a preliminary report. *Am J Ophthalmol.* 1954; 37:13–15. [PubMed: 13114318]
23. Ondetti MA, Cushman DW. Azetidine-2-carboxylic acid derivatives. 1977 US 4046889.
24. Mordini A, Massaro A, Reginato G, Russo F, Taddei M. Microwave-Assisted Transformation of Esters into Hydroxamic Acids. *Synthesis.* 2007; 20:3201–3204.
25. Richon VM, Webb Y, Merger R, Sheppard T, Jursic B, Ngo L, Civoli F, Breslow R, Rifkind RA, Marks PA. Second generation hybrid polar compounds are potent inducers of transformed cell differentiation. *Proc Natl Acad Sci U S A.* 1996; 93:5705–5708. [PubMed: 8650156]
26. Carter GW, Young PR, Albert DH, Bouska J, Dyer R, Bell RL, Summers JB, Brooks DW. 5-lipoxygenase inhibitory activity of zileuton. *J Pharmacol Exp Ther.* 1991; 256:929–937. [PubMed: 1848634]
27. Agrawal A, DeSoto J, Fullagar JL, Maddali K, Rostami S, Richman DD, Pommier Y, Cohen SM. Probing chelation motifs in HIV integrase inhibitors. *Proc Natl Acad Sci U S A.* 2012; 109:2251–2256. [PubMed: 22308350]
28. Boldt GE, Kennedy JP, Janda KD. Identification of a Potent Botulinum Neurotoxin A Protease Inhibitor Using in Situ Lead Identification Chemistry. *Org Lett.* 2006; 8:1729–1732. [PubMed: 16597152]
29. Tamura Y, Watanabe F, Nakatani T, Yasui K, Fuji M, Komurasaki T, Tsuzuki H, Maekawa R, Yoshioka T, Kawada K, Sugita K, Ohtani M. Highly Selective and Orally Active Inhibitors of Type IV Collagenase (MMP-9 and MMP-2): □ N-Sulfonylamino Acid Derivatives. *J Med Chem.* 1998; 41:640–649. [PubMed: 9484512]
30. Agrawal A, Romero-Perez D, Jacobsen JA, Villarreal FJ, Cohen SM. Zinc-Binding Groups Modulate Selective Inhibition of MMPs. *ChemMedChem.* 2008; 3:812–820. [PubMed: 18181119]
31. MacPherson LJ, Bayburt EK, Capparelli MP, Carroll BJ, Goldstein R, Justice MR, Zhu L, Hu Si, Melton RA, Fryer L, Goldberg RL, Doughty JR, Spirito S, Blancuzzi V, Wilson D, O'Byrne EM, Ganu V, Parker DT. Discovery of CGS 27023A, a Non-Peptidic, Potent, and Orally Active Stromelysin Inhibitor That Blocks Cartilage Degradation in Rabbits. *J Med Chem.* 1997; 40:2525–2532. [PubMed: 9258358]
32. Yawalkar SJ. Milestones in the research and development of desferrioxamine. *Nephrol Dial Transpl.* 1993; 8:40–42.
33. Meldrum NU, Roughton FJW. Carbonic anhydrase. Its preparation and properties. *J Physiol.* 1933; 80:113–142. [PubMed: 16994489]

34. Johnson JL, Devel L, Czarny B, George SJ, Jackson CL, Rogakos V, Beau F, Yiotakis A, Newby AC, Dive V. A Selective Matrix Metalloproteinase-12 Inhibitor Retards Atherosclerotic Plaque Development in Apolipoprotein E–Knockout Mice. *Arterioscler Thromb Vasc Biol.* 2011;528–535. [PubMed: 21212406]
35. Devel L, Rogakos V, David A, Makaritis A, Beau F, Cuniasse P, Yiotakis A, Dive V. Development of Selective Inhibitors and Substrate of Matrix Metalloproteinase-12. *J Biol Chem.* 2006; 281:11152–11160. [PubMed: 16481329]
36. Dufour A, Overall CM. Missing the target: matrix metalloproteinase antitargets in inflammation and cancer. *Trends Pharmacol Sci.* 2013; 34:233–242. [PubMed: 23541335]
37. Lovejoy B, Welch AR, Carr S, Luong C, Broka C, Hendricks RT, Campbell JA, Walker KA, Martin R, Van Wart H, Browner MF. Crystal structures of MMP-1 and -13 reveal the structural basis for selectivity of collagenase inhibitors. *Nat Struct Mol Biol.* 1999; 6:217–221.
38. Major Jourden JL, Cohen SM. Hydrogen Peroxide Activated Matrix Metalloproteinase Inhibitors: A Prodrug Approach. *Angew Chem Intl Ed.* 2010; 122:6947–6949.
39. Caton J, Ryan ME. Clinical studies on the management of periodontal diseases utilizing subantimicrobial dose doxycycline (SDD). *Pharmacol Res.* 2011; 63:114–120. [PubMed: 21182947]
40. Ramirez-Sanchez M, Prieto I, Wangenstein R, Banegas I, Segarra AB, Villarejo AB, Vives F, Cobo J, de Gasparo M. The Renin-Angiotensin System: New Insight into Old Therapies. *Curr Med Chem.* 2013; 20:1313–1322. [PubMed: 23409710]
41. Patchett AA, Harris E, Tristram EW, Wyvratt MJ, Wu MT, Taub D, Peterson ER, Ikeler TJ, Broeke J, Payne LG, Ondeyka DL, Thorsett ED, Greenlee WJ, Lohr NS, Hoffsommer RD, Joshua H, Ruyle WV, Rothrock JW, Aster SD, Maycock AL, Robinson FM, Hirschmann R, Sweet CS, Ulm EH, Gross DM, Vassil TC, Stone CA. A new class of angiotensin-converting enzyme inhibitors. *Nature.* 1980; 288:280–283. [PubMed: 6253826]
42. Kast RE, Halatsch ME. Matrix Metalloproteinase-2 and -9 in Glioblastoma: A Trio of Old Drugs—Captopril, Disulfiram and Nelfinavir—Are Inhibitors with Potential as Adjunctive Treatments in Glioblastoma. *Arch Medical Res.* 2012; 43:243–247.
43. Espin JC, Wichers HJ. Effect of captopril on mushroom tyrosinase activity in vitro. *Biochim Biophys Acta.* 2001; 1544:289–300. [PubMed: 11341938]
44. Marks PA, Dokmanovic M. Histone deacetylase inhibitors: discovery and development as anticancer agents. *Expert Opin Invest Drugs.* 2005; 14:1497–1511.
45. Dokmanovic M, Clarke C, Marks PA. Histone deacetylase inhibitors: overview and perspectives. *Mol Cancer Res.* 2007; 5:981–989. [PubMed: 17951399]
46. Dey P. Chromatin Remodeling, Cancer and Chemotherapy. *Curr Med Chem.* 2006; 13:2909–2919. [PubMed: 17073637]
47. Grant S, Easley C, Kirkpatrick P. Vorinostat. *Nat Rev Drug Discovery.* 2007; 6:21–22.
48. Bertino EM, Otterson GA. Romidepsin: a novel histone deacetylase inhibitor for cancer. *Expert Opin Invest Drugs.* 2011; 20:1151–1158.
49. Kim YJ, Uyama H. Tyrosinase inhibitors from natural and synthetic sources: structure, inhibition mechanism and perspective for the future. *Cell Mol Life Sci.* 2005; 62:1707–1723. [PubMed: 15968468]
50. Ubeid AA, Do S, Nye C, Hantash BM. Potent low toxicity inhibition of human melanogenesis by novel indole-containing octapeptides. *Biochim Biophys Acta.* 2012; 1820:1481–1489. [PubMed: 22609875]
51. Ismaya WT, Rozeboom HJ, Weijn A, Mes JJ, Fusetti F, Wichers HJ, Dijkstra BW. Crystal structure of *Agaricus bisporus* mushroom tyrosinase: identity of the tetramer subunits and interaction with tropolone. *Biochemistry.* 2011; 50:5477–5486. [PubMed: 21598903]
52. Fullagar JL, Garner AL, Struss AK, Day JA, Martin DP, Yu J, Cai X, Janda KD, Cohen SM. Antagonism of a zinc metalloprotease using a unique metal-chelating scaffold: tropolones as inhibitors of *P. aeruginosa* elastase. *Chemical Communications.* 2013; 49:3197–3199. [PubMed: 23482955]
53. Huebers HA, Finch CA. The physiology of transferrin and transferrin receptors. *Phys Rev.* 1987; 67:520–582.

54. Abergel RJ, Zawadzka AM, Raymond KN. Petrobactin-Mediated Iron Transport in Pathogenic Bacteria: Coordination Chemistry of an Unusual 3,4-Catecholate/Citrate Siderophore. *J Am Chem Soc.* 2008; 130:2124–2125. [PubMed: 18220393]
55. Pollack S, Aisen P, Lasky FD, Vanderhoff G. Chelate mediated transfer of iron from transferrin to desferrioxamine. *Br J Haematol.* 1976; 34:231–235. [PubMed: 974036]
56. Marques HM, Watson DL, Egan TJ. Kinetics of Iron Removal from Human Serum Monoferric Transferrins by Citrate. *Inorg Chem.* 1991; 30:3758–3762.
57. Turcot I, Stintzi A, Xu J, Raymond KN. Fast biological iron chelators: kinetics of iron removal from human diferric transferrin by multidentate hydroxypyridonates. *J Biol Inorg Chem.* 2000; 5:634–641. [PubMed: 11085654]
58. Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE. The Protein Data Bank. *Nucleic Acids Research.* 2000; 28:235–242. [PubMed: 10592235]
59. Dhanaraj V, Ye QZ, Johnson LL, Hupe DJ, Ortwine DF, Dunbar JJB, Rubin JR, Pavlovsky A, Humblet C, Blundell TL. X-ray structure of a hydroxamate inhibitor complex of stromelysin catalytic domain and its comparison with members of the zinc metalloproteinase superfamily. *Structure.* 1996; 4:375–386. [PubMed: 8740360]
60. Avvaru BS, Kim CU, Sippel KH, Gruner SM, Agbandje-McKenna M, Silverman DN, McKenna R. A short, strong hydrogen bond in the active site of human carbonic anhydrase II. *Biochemistry.* 2010; 49:249–51. [PubMed: 20000378]
61. Bressi JC, Jennings AJ, Skene R, Wu Y, Melkus R, De Jong R, O'Connell S, Grimshaw CE, Navre M, Gangloff AR. Exploration of the HDAC2 foot pocket: Synthesis and SAR of substituted N-(2-aminophenyl)benzamides. *Bioorg Med Chem Lett.* 2010; 20:3142–3145. [PubMed: 20392638]
62. Natesh R, Schwager SL, Sturrock ED, Acharya KR. Crystal structure of the human angiotensin-converting enzyme-lisinopril complex. *Nature.* 2003; 421:551–554. [PubMed: 12540854]
63. Engelman A, Cherepanov P. The structural biology of HIV-1: mechanistic and therapeutic insights. *Nat Rev Microbiol.* 2012; 10:279–290. [PubMed: 22421880]
64. Summa V, Petrocchi A, Bonelli F, Crescenzi B, Donghi M, Ferrara M, Fiore F, Gardelli C, Gonzalez Paz O, Hazuda DJ, Jones P, Kinzel O, Laufer R, Monteagudo E, Muraglia E, Nizi E, Orvieto F, Pace P, Pescatore G, Scarpelli R, Stillmock K, Witmer MV, Rowley M. Discovery of Raltegravir, a Potent, Selective Orally Bioavailable HIV-Integrase Inhibitor for the Treatment of HIV-AIDS Infection. *J Med Chem.* 2008; 51:5843–5855. [PubMed: 18763751]
65. Aparoy P, Kumar Reddy K, Reddanna P. Structure and Ligand Based Drug Design Strategies in the Development of Novel 5- LOX Inhibitors. *Curr Med Chem.* 2012; 19:3763–3778. [PubMed: 22680930]
66. Silverman ES, Drazen JM. The Biology of 5-Lipoxygenase: Function, Structure, and Regulatory Mechanisms. *Proc Assoc Am Physicians.* 1999; 111:525–536. [PubMed: 10591081]
67. Jacobsen JA, Fullagar JL, Miller MT, Cohen SM. Identifying Chelators for Metalloprotein Inhibitors Using a Fragment-Based Approach. *J Med Chem.* 2010; 54:591–602. [PubMed: 21189019]
68. Martin DP, Cohen SM. Nucleophile recognition as an alternative inhibition mode for benzoic acid based carbonic anhydrase inhibitors. *Chem Commun.* 2012; 48:5259–5261.
69. Abdul-Hay SO, Lane AL, Caulfield TR, Claussin C, Bertrand J, Masson A, Choudhry S, Fauq AH, Maharvi GM, Leissring MA. Optimization of peptide hydroxamate inhibitors of insulin-degrading enzyme reveals marked substrate-selectivity. *J Med Chem.* 2013; 56:2246–2255. [PubMed: 23437776]
70. Liu J, Yi W, Wan Y, Ma L, Song H. 1-(1-Arylethylidene)thiosemicarbazide derivatives: a new class of tyrosinase inhibitors. *Bioorg Med Chem.* 2008; 16:1096–1102. [PubMed: 18326070]
71. Hamilton DH, Turcot I, Stintzi A, Raymond KN. Large cooperativity in the removal of iron from transferrin at physiological temperature and chloride ion concentration. *J Biol Inorg Chem.* 2004; 9:936–944. [PubMed: 15517438]
72. Ohkanda J, Kamitani J, Tokumitsu T, Hida Y, Konakahara T, Katoh A. Novel Iron Sequestering Agents: Synthesis and Iron-Chelating Properties of Hexadentate Ligands Composed of 1-Hydroxy-2(1H)-pyrimidinone, w-Amino Carboxylic Acid, and Tris(2-aminoethyl)amine. *J Org Chem.* 1997; 62:3618–3624.

73. Rubin EH, Agrawal NGB, Friedman EJ, Scott P, Mazina KE, Sun L, Du L, Ricker JL, Frankel SR, Gottesdiener KM, Wagner JA, Iwamoto M. A Study to Determine the Effects of Food and Multiple Dosing on the Pharmacokinetics of Vorinostat Given Orally to Patients with Advanced Cancer. *Clin Cancer Res.* 2006; 12:7039–7045. [PubMed: 17145826]
74. Yano I, Takayama A, Takano M, Inatani M, Tanihara H, Ogura Y, Honda Y, Inui K. Pharmacokinetics and pharmacodynamics of acetazolamide in patients with transient intraocular pressure elevation. *Eur J Clin Pharmacol.* 1998; 54:63–68. [PubMed: 9591933]
75. Richer C, Giroux B, Plouin PF, Maarek B, Giudicelli JF. Captopril: pharmacokinetics, antihypertensive and biological effects in hypertensive patients. *Brit J Clin Pharmacol.* 1984; 17:243–250.
76. Lee P, Mohammed N, Marshall L, Abeysinghe RD, Hider RC, Porter JB, Singh S. Intravenous infusion pharmacokinetics of desferrioxamine in thalassaemic patients. *Drug Metab Dispos.* 1993; 21:640–644. [PubMed: 8104123]
77. Dubé L, Swanson L, Awni W. Zileuton, a leukotriene synthesis inhibitor in the management of chronic asthma. *Clin Rev Allerg Immun.* 1999; 17:213–221.
78. Rae TD, Schmidt PJ, Pufahl RA, Culotta VC, O'Halloran TV. Undetectable Intracellular Free Copper: The Requirement of a Copper Chaperone for Superoxide Dismutase. *Science.* 1999; 284:805–808. [PubMed: 10221913]
79. Outten CE, O'Halloran TV. Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. *Science.* 2001; 292:2488–3492. [PubMed: 11397910]
80. Thorp HH, O'Halloran TV. Screening for modulators of metalation pathways for metalloproteins. 2007 AU 2006/304860 A1.
81. Monnard FW, Heinisch T, Nogueira ES, Schirmer T, Ward TR. Human Carbonic Anhydrase II as a host for piano-stool complexes bearing a sulfonamide anchor. *Chem Commun.* 2011; 47:8238–8240.

Abbreviations

ACE	angiotensin converting enzyme
BoNT/A	botulinum neurotoxin serotype A
BOTI	botulinum neurotoxin inhibitor
hCAII	human carbonic anhydrase II
HDAC-2	histone deacetylase 2
HOPO	hydroxypyridinone
LOX	lipoxygenase
MBG	metal-binding group
MMP	matrix metalloproteinase
RCD-1	raltegravir chelator derivative 1
SAHA	suberoylanilide hydroxamic acid
TLCK	tosyl lysine chloromethyl ketone
TPCK	tosyl phenylalanyl chloromethyl ketone
TSA	trichostatin A
TY	tyrosinase

Figure 2. Reactions catalyzed by and active sites of the metalloenzymes studied (top to bottom):⁵⁸ MMP-2 (1QIB),⁵⁹ hCAII (3KS3),⁶⁰ HDAC-2 (3MAX),⁶¹ ACE (1O8A),⁶² and TY (2Y9W).⁵¹ Residues involved in ligating the metal ion are explicitly shown.

Figure 3. Percent enzyme activity of MMP-2 (black) and MMP-12 (gray) in the presence of 10 μ M of each metalloenzyme inhibitor.

Figure 4. Percent enzyme activity of hCAII in the presence of 10 μ M of each metalloenzyme inhibitor.

Figure 5. Percent enzyme activity of HDAC-2 in the presence of 10 μ M of each metalloenzyme inhibitor.

Figure 6.
Percent enzyme activity of ACE in the presence of 10 μ M of each metalloenzyme inhibitor.

Figure 7.
Percent enzyme activity of TY in the presence of 10 μ M of each metalloenzyme inhibitor.

Figure 8.
Percent enzyme activity of trypsin in the presence of 10 μ M of each metalloenzyme inhibitor.

Figure 9. Percent enzyme activity for each metalloenzyme in the presence of 10 μ M captopril (black) or CGS (gray) highlighting the selectivity of each inhibitor.

Table 1

Metalloenzyme inhibitors examined in this study.

Inhibitor	Source	Target Protein	IC₅₀ (nM)	Indication	FDA Approved
Acetazolamide	Commercial ²²	hCAII	25	Glaucoma	Yes
Captopril	Commercial ²³	ACE	21	Hypertension Cutaneous T-	Yes
SAHA	Synthesized ^{24, 25}	HDAC	10	cell lymphoma	Yes
Zileuton	Commercial ²⁶	5-LOX	600	Asthma	Yes
RCD-1	Synthesized ²⁷	HIV IN	60	HIV/AIDS	No
BoTI	Synthesized ²⁸	BoNT/A	410	Botulism	No
NSA	Synthesized ²⁹	MMP-2, -9	240, 310	Tumor malignancy	No
1,2-HOPO-2	Synthesized ³⁰	MMP-12	18	Tumor malignancy	No
CGS	Synthesized ³¹	MMPs	8-43	Tumor malignancy	No
Deferoxamine	Commercial ³²	Transferrin/Ferritin	N/A	Iron overload	Yes

Table 2

Pseudo-first-order rate constants for iron removal. 100 μ M Human holo-transferrin and 1 mM of each compound is used in the assay.

Inhibitor	$k_{\text{obs}} (\times 10^2 \text{ min}^{-1})$
Acetazolamide	0.00
Captopril	0.00
SAHA	0.00
Zileuton	0.00
RCD-1	-
BOTI	0.00
NSA	0.00
1,2-HOPO-2	0.92
CGS	0.00
Deferoxamine	0.00
Deferiprone	4.06