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Investigating the Selectivity of Metalloenzyme Inhibitors in the Presence of Competing Metalloproteins

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Abstract

Metalloprotein inhibitors (MPi) are an important class of therapeutics for the treatment of a variety of diseases, including hypertension, cancer, and HIV/AIDS. However, despite their clinical success, there is an apprehension that MPi may be less selective than other small molecule therapeutics, and more prone to inhibit off-target metalloenzymes. We have examined the issue of MPi specificity by investigating the selectivity of a variety of MPi against a representative panel of metalloenzymes in the presence of competing metalloproteins (metallothionein, myoglobin, carbonic anhydrase, and transferrin). Our findings reveal that a wide variety of MPi do not exhibit a reduction in inhibitory activity in the presence of large excesses of competing metalloproteins, suggesting that the competing proteins do not titrate the MPi away from its intended target. This study represents a rudimentary, but important means to mimic the biological milieu where other metalloproteins are present that could compete the MPi away from its target. The strategy used here may serve as a useful approach to examining the selectivity of other MPi in development.

Keywords

metalloprotein; inhibitor; selectivity; competing protein; metalloenzyme

Introduction

Metalloproteins, which contain metal ion cofactors at their active site, represent a broad class of validated clinical targets. Over 30% of the human proteome consists of metalloenzymes, which execute a variety of biological functions, such as matrix degradation, DNA transcription, blood pH homeostasis, and many others.^[1] Misregulation of several metalloenzymes has been implicated in a wide range of diseases.^[2] Metalloprotein inhibitors (MPi) offer an appealing approach to develop therapeutics for the treatment of a variety of ailments, including hypertension, bacterial and viral infections, and cancer, thus having a significant impact on improving human health.^[3] However, despite their clinical success, there exists a common apprehensions that MPi are less selective than other small molecule therapeutics, and thus more prone to inhibit off-target metalloenzymes raising concerns about their safety. There is a perception that MPi indiscriminately inhibit

all metalloenzymes or that they strip the catalytic metal ion from off-target metalloproteins.^[4] Although the potential for these issues is frequently raised, few studies have addressed the validity of these concerns.^[5] Our group recently reported on the selectivity of MPi by evaluating the activity of seven metalloenzymes against a panel of nine MPi and one metal-sequestering agent (deferoxamine).^[5] These findings demonstrated that the MPi do not show off-target activity, even at concentrations far above the IC₅₀ value against their respective targets. These results prompted us to pursue a more rigorous examination of MPi specificity by investigating the selectivity of a variety of MPi against a panel of metalloenzymes in the presence of competing metalloproteins, including metallothionein, carbonic anhydrase, myoglobin, and transferrin. This selection of competing proteins are relatively abundant and represent different classes (e.g. intracellular and extracellular enzymes) of metalloenzymes that play key roles in many biological processes (e.g. oxygen transport, metal ion trafficking and homeostasis, etc.). Therefore, our efforts here represent a simplistic attempt to better mimic a complex milieu where other metalloproteins are present that could interact with an MPi and compete for binding over the desired target. This study is analogous to conventional enzyme assays that are performed in the presence of a plasma protein (e.g. BSA) to evaluate off-target binding mediated via non-specific hydrophobic interactions.^[6] Here we seek to address these critical questions surrounding MPi selectivity, and determine whether competing proteins will modulate the specificity of MPi.

Results and Discussion

Selection of inhibitors, targets, and competing proteins

Typically, metalloprotein inhibitors contain a metal-binding pharmacophore (MBP) that directly binds to the catalytic metal ion of the target protein.^[7] In this study, five compounds (Figure 1, Table S1) were evaluated, which represent a variety of metalloenzyme inhibitors with a diverse range of MBPs (5 distinct MBPs) and protein targets (HDAC-1, HDAC-6, MMP-2, MMP-12, and hCAII). In addition, four competing proteins, metallothionein (MT), carbonic anhydrase (CA), myoglobin (Mb), and transferrin (Tf) were selected for this study based on their broad distribution (CA and Mb) or key role in metal ion trafficking and homeostasis (MT and Tf). A brief description of the MPi, their targets, and the competing proteins is provided below.

Histone deacetylases (HDACs) represent one important family of Zn(II)-dependent metalloenzymes that play a critical role in gene expression by reversing the regulatory acetylation of histone proteins.^[8] Discovered by Richon et al in 1996,^[9] SAHA (suberoylanilide hydroxamic acid, Vorinostat, Merck) is a FDA approved, broad spectrum HDAC inhibitor for the treatment of cutaneous T-cell lymphoma. Matrix metalloproteinases (MMPs) are another group of Zn(II)-dependent metalloenzymes, which are involved in maintenance of extracellular matrix components.^[10] MMPs are reported to disrupt normal angiogenesis in malignant tumors and thus constitute prototypical metalloenzyme targets.^[11] Three MMP inhibitors (Figure 1) were chosen for this study based on their different MBPs as well as known isoform selectivity. NSA (N-sulfonylamino acid) is an MMP-2 and MMP-9 selective inhibitor (IC₅₀ values of 240 and 310 nM, respectively) that contains a

carboxylic acid moiety as the MBP. CGS 27023A^[12] is a broad-spectrum compound that incorporates the common hydroxamic acid MBP. 1,2-HOPO-2, using a hydroxypyridinone MBP, is a semi-selective MMP-12 inhibitor, with some activity against other isoforms.^[13] Finally, carbonic anhydrases (CAs, Figure 2) are one of the oldest known classes of Zn(II) metalloenzyme.^[14] They are involved in catalytic dehydration of bicarbonate, regulation of blood pH and CO₂ homeostasis, and ion transport.^[15] An early FDA-approved human carbonic anhydrase (hCA) inhibitor, Acetazolamide (Diamox), has proven to be effective in the treatment of glaucoma.^[16]

With respect to the competing metalloproteins studied, three distinct systems were examined. Metallothioneins (MT) are a family of ubiquitous metal binding proteins, which play a critical role in the regulation of homeostasis of cellular metal ions (e.g. Cu, Zn) in the intracellular space.^[17] MTs contain cysteine residues which allow MTs to bind up to 7 Zn ions (Figure 2).^[18] The second competing protein selected was myoglobin (Mb, Figure 2), a monomeric, prototypical hemoprotein that has a globular structure.^[19] Myoglobin is the primary oxygen-carrying component of muscle,^[20] and is among the most well studied metalloproteins in biology. Another competing protein used was transferrin (Tf, Figure 2), an iron-binding protein that facilitates cellular iron uptake and transport,^[21] which represents an important and exchangeable metal ion pool in the bloodstream.^[22] Tf is also of great interest as a potential anticancer drug carrier.^[23] Finally, hCAII (Figure 2), one of the target proteins, was also used as a competing protein in some experiments.

Metalloprotein screening experiments with one competing protein. Initial metalloprotein screening experiments (hCAII, MMP-2, MMP-12, HDAC-1, and HDAC-6) were performed with holometallothionein-1 (rabbit liver MT, 7 Zn per monomer, Enzo Life Sciences) as a competing metalloprotein (Figure 3–5). All assays were validated in the presence of excess MT to determine the maximum concentrations of MT that can be combined with each target protein without detectable interference with the native metalloenzyme activity or assay readout in the absence of inhibitor (data not shown). It is important to note that the concentrations of competing protein used in this study are comparable or higher than the reported concentrations of BSA (~1 μ M) frequently used in conventional enzyme assays to evaluate off-target, hydrophobic binding.^[6] Once validated, the various MPi were tested for inhibitory activity, where an increase in IC₅₀ value would be indicative of off-target metalloprotein interference. As described below, in all of the systems evaluated, the MPi exhibited no reduction in inhibitory activity against their target protein in the presence of large excesses of MT, suggesting that the competing protein did not titrate MPi away from its target.

In order to evaluate the influence of MT on the inhibitory activity of acetazolamide against hCAII (10 nM), the esterase activity of hCAII with *p*-nitrophenyl acetate as a substrate^[24] was monitored upon the addition of inhibitor (25 nM) and various concentrations of MT (Figure 3). The target and competing proteins were preincubated with the inhibitor at 30 °C for 10 min before addition of substrate. As shown in Figure 3, even upon addition of 1250 nM of MT (50 equiv over the concentration of acetazolamide and 125 equiv over the concentration of hCAII), the enzymatic activity of hCAII remains at the expected ~50% in the presence of 25 nM of acetazolamide. The results show that the large excess of competing

protein does not titrate inhibitor away from its target, even though both the target and competitor proteins are Zn metalloproteins. Similarly, the inhibition of MMP-2 (2.7 nM) by CGS (3 nM) or NSA (30 nM) in the presence of various concentrations of MT revealed both CGS and NSA maintain their specificity in the presence of a large excess of MT (up to 50 equiv over the concentration of MPi, Figure S1). To further verify these results, an even larger excess of MT (up to 9 μ M of MT, 300 equiv over the concentration of 1,2-HOPO-2, and 13235 equiv over the concentration of MMP-12) was added in competition with MMP-12 (0.68 nM), prior to the treatment with 1,2-HOPO-2 (30 nM, the IC₅₀ of 1,2-HOPO-2 for MMP-12). The results revealed that the addition of this large excess of MT did not perturb the activity of 1,2-HOPO-2 on MMP-12 (Figure 3), which validated that MT exhibits no interference with the specificity of this MPi.

The inhibition of HDAC-1 (0.25 μ M) and HDAC-6 (0.16 μ M) by SAHA near its IC₅₀ for each protein has also been evaluated in the presence of various concentration of MT. As shown in Figure 4, no substantial decrease in SAHA activity was observed, indicating the MPi was not titrated away from its HDAC targets. It is notable that there was a slight decrease (as much as 10% loss) in HDAC activity at the highest concentrations of MT (>500 nM) used, but this occurred even in the absence of MPi (Figure S2). This indicates the slight loss in SAHA activity at the highest concentrations of MT are due to direct interactions/perturbations of MT against the HDACs, and not via titration of SAHA away from the HDAC targets per se.

To broaden the scope of this study and verify our observations with MT, other representative metalloproteins, including myoglobin (Mb), transferrin (Tf), and hCAII were also assessed in competition with the target proteins hCAII and MMP-2. hCAII (10 nM) activity in the presence of acetazolamide (25 nM) showed no change in inhibitory activity using increasing concentrations of Mb or Tf (Figure 5 and Figure S5, up to 50 equiv over acetazolamide and up to 125 equiv over hCAII). Similar results were also observed for MMP-2 inhibited by either NSA (30 nM) or CGS (3 nM). In the case of MMP-2, neither Mb, Tf, nor hCAII as a competing protein (Figure S3, S4, up to 166 equiv over CGS, up to 50 equiv over NSA and up to 555 equiv over MMP-2) had any effect on inhibition, suggesting these competing proteins are not titrating the MPi away from the MMP-2 target. These results substantiate that MPi show excellent target specificity in the presence of a diverse array of competing metalloproteins.

Inhibition in the presence of multiple competing proteins

As a crude means to recapitulate the biological milieu, where many metalloproteins are present that could compete for MPi binding, we conducted screening assays in the presence of combinations of several competing proteins (MT, Mb, Tf, and hCAII). Using the enzyme/inhibitor combination of MMP-2 with CGS as a representative case, enzyme activity was evaluated in the presence of various combinations of competing metalloproteins. Each competitor protein was present at a concentration of 400 nM, which is >130 equivalents over the concentrations of CGS (3 nM) and MMP-2 (2.7 nM). Regardless of the number or nature of competing proteins included in the assay, no drop in inhibition was observed, indicating that CGS remained selective for MMP-2 even in the presence of a large excess of several

competing metalloprotein combinations (Figure 6). A similar experiment, using hCAII and acetazolamide as the target/MPI combination provided comparable results. Combinations of competing proteins (1 μ M each, which is 40 equiv over the concentration of acetazolamide (25 nM), and 100 equiv over the concentration of hCAII), gave no change in the activity of acetazolamide against hCAII, suggesting no titration of the MPI away from its target by the various combinations of competing proteins (Figure S6).

Conclusions

The specificity of a broad range of MPI has been evaluated in the presence of a large excess of competing metalloproteins or combination of competing metalloproteins, which is a simplistic representation of the biological milieu. Our finding revealed that competing proteins will not interfere with the specificity of an MPI. All of the tested inhibitors show excellent selectivity in the presence of large excess of competing metalloproteins. Competing metalloproteins were present in concentrations comparable to or higher than typically used for other competitors, such as BSA. Metalloprotein screening experiments with one or multiple competing metalloproteins demonstrated that MPI exhibited no reduction in inhibitory activity in the presence of a large excesses of competing proteins (up to 300 equivalents of competing metalloprotein over the concentration of MPI), suggesting that the competing protein does not titrate the MPI away from its target, and MPI maintain their specificity against the target metalloproteins. This study is significant in addressing a core concern surrounding the specificity of MPI, and represents a rudimentary means to provide a 'BSA-equivalent' to non-specific binding for metalloprotein assays. By conducting the evaluation of MPI under these conditions, the results validate that the development of MPI present no more risk for nonspecific activity than small molecule inhibitors of metal-independent enzymes, which should help to alleviate concerns about pursuing metalloproteins as valuable targets for addressing therapeutic needs.

Experimental Section

Materials

All reagents and acetazolamide,^[16] were obtained from Sigma-Aldrich. 1,2-HOPO-2,^[25] CGS^[12] and SAHA^[26] were prepared by literature methods. Absorbance assays were performed using a BioTek Synergy HT microplatereader. Fluorescence assays were performed using either a BioTek FLx800 microplate reader or a BioTek Synergy HT microplate reader. MMP-2, MMP-12, HDAC-1, HDAC-6 and metallothionein were obtained from Enzo Life Sciences (Farmingdale, NY). Human carbonic anhydrase, myoglobin and transferrin were obtained from Sigma-Aldrich.

MMP-2/MMP-12 Assays.^[5]

MMP-2 and -12, and OmniMMP fluorogenic substrate were purchased from Enzo Life Sciences (Farmingdale, NY). The assays were carried out in black NUNC 96-well plates. Each well contained a volume of 100 μ L including buffer (50 mM HEPES, 10mM CaCl_2 , 0.05% Brij-35, pH 7.5), human recombinant MMP (1.16 U of MMP-2 or 0.035 U of MMP-12, ENZO Life Sciences), inhibitor (at the IC50), fluorogenic OmniMMP substrate (4

μM Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂-AcOH, ENZO Life Sciences), and various concentration of competing proteins. The mixture of proteins (either MMPs or MMPs and competing proteins) and inhibitor were preincubated in solution at 37 °C for 30 min, followed by the addition of the substrate to initiate the reaction. The highest concentration of MT used in each MMP assay was based on the maximum concentrations of MT achievable that did not interfere with the assay readout. For the MMP-2 assay the MT concentration could be as high as 1.5 μM , while the highest concentration of MT used in MMP-12 assay was 9 μM . The change in fluorescence was monitored for 30 min by either BioTek Flx 800 or BioTek synergy HT fluorescence plate reader with excitation and emission wavelengths at 320 and 400 nm, respectively. The 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/competing protein combination was conducted three times. The data were normalized to values measured for uninhibited enzyme. Assays were reported as mean $\pm$ standard deviation.

HDAC-1/HDAC-6 Assays

The HDAC assay kit was purchased from BPS Bioscience (San Diego, CA). The assays were carried out in black NUNC 96-well plates. Each well contained a volume of 50 μL including buffer (BPS Bioscience, catalogue no. 50031), the mixture of proteins (HDAC-1 (0.033 mg/ml, BPS Bioscience, catalogue no. 50051) or HDAC-6 (0.025 mg/ml, BPS Bioscience, catalogue no. 50006) and competing proteins), inhibitor (at the IC₅₀), BSA (1 mg/mL, Sigma-Aldrich), and HDAC substrate 3 (20 μM , BPS Bioscience, catalogue no. 50037). Upon addition of substrate, the plate was incubated at 37 °C for 30 min. HDAC assay developer (50 μL , BPS Bioscience, catalogue no. 50030) was then added to each well and the plate incubated for 15 min at room temperature. The fluorescence was recorded at excitation and emission wavelengths of 360 and 460 nm, respectively. Blank wells containing no inhibitor or protein were subtracted from all wells. The control wells, containing no inhibitor, were arbitrarily set as 100% activity. The assays were performed in triplicate, and each assay contained each inhibitor/competing protein combination was conducted three times. The data were normalized to values measured for uninhibited enzyme. Assays were reported as mean $\pm$ standard deviation.

hCAII Assays.^[24]

Human Carbonic AnhydraseII Assays were carried out in clear Costar 96-well plates. Each well contained a volume of 100 μL including buffer (50 mM HEPES buffer, pH = 8.0), a mixture of proteins (10 nM hCAII and various concentrations of competing proteins), inhibitor (at IC₅₀ value), and substrate *p*-nitrophenyl acetate (500 μM). The protein mixture and inhibitor were incubated in solution at 30 °C for 10 min prior to the addition of the *p*-nitrophenyl acetate to initiate the reaction. The change in absorbance was monitored at 405 nm for 15 min. 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 containing each inhibitor/competing protein combination was conducted three times. The data was

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Metalloprotein inhibitors evaluated in this study. Metal-binding pharmacophores (MBPs) are highlighted in boxes.

Figure 2. Structure of proteins involved in this research. (a) MMP-12 (Zn-dependent, PDB ID 2OXU), (b) HDAC-7 (Zn-dependent, PDB ID 3C0Y), (c) hCAII (Zn-dependent, PDB ID 4JS6), (d) myoglobin (Fe-heme dependent, PDB 3LR7), (e) metallothionein-2 (Zn transport, α domain PDB ID 1MHU, β domain PDB ID 2MHU) and (f) transferrin (non-heme Fe transport, PDB ID 1A8E). Target proteins are shown as blue ribbons and competing proteins are shown as green ribbons, including hCAII, which served as both a target and competitor. Metal ions shown in spacefilling and ligating groups shown as sticks.

Figure 3.

Top: Percent enzyme activity remaining for hCAII (10 nM) in the presence of 25 nM acetazolamide ($IC_{50} = 25$ nM) and increasing concentrations of MT. Bottom: Percent enzyme activity remaining for MMP-12 (0.68 nM) in the presence of 30 nM 1,2-HOPO-2 ($IC_{50} = 30$ nM) and increasing concentrations of MT. The ratios of MPi to MT are indicated above each column. Error bars represent the $\pm$ SD of three independent replicates ($n=3$).

Figure 4.

Percent enzyme activity remaining for HDAC-1 (top, 0.25 μ M) and HDAC-6 (bottom, 0.16 μ M) in the presence of 53 or 22 nM SAHA (IC_{50} = 53 nM and 22 nM, respectively) and increasing concentrations of MT. The ratios of MPi to MT are indicated above each column. Error bars represent the $\pm$ SD of three independent replicates ($n=3$).

Figure 5. Percent enzyme activity remaining for hCAII (10 nM) in the presence of 25 nM acetazolamide ($IC_{50} = 25$ nM) and increasing concentrations of Tf. The ratios of MPi to Tf are indicated above each column. Error bars represent the $\pm SD$ of three independent replicates ($n=3$).

Figure 6. Enzymatic activity of MMP-2 (2.7 nM) inhibited by CGS (3 nM) in the presence of varying combinations of competing proteins (400 nM each). Error bars represent the $\pm$ SD of three independent replicates ($n=3$).