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Masking thiol reactivity with thioamide, thiourea, and thiocarbamate-based MBPs

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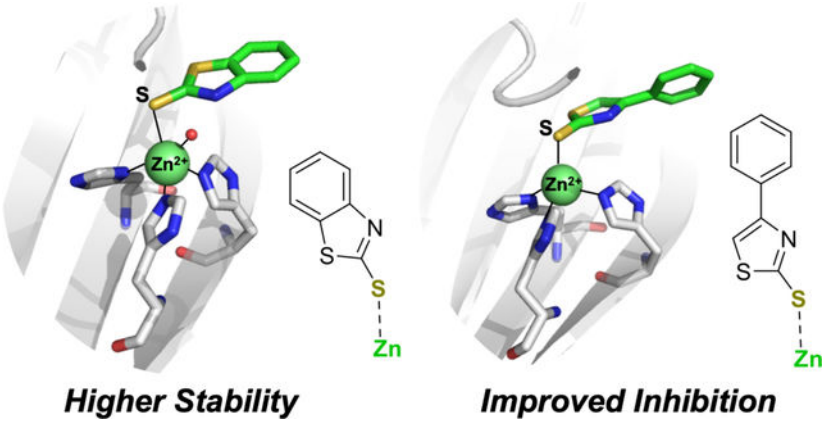
Abstract

Thioamides, thioureas, and thiocarbamates are introduced as stable, sulfur-based metal-binding pharmacophores (MBPs) for use in metalloenzyme fragment-based drug discovery (mFBDD). MBP reactivity, bioactivity, and structural studies show that these molecules can act as ligands for Zn(II)-dependent metalloenzymes including human carbonic anhydrase II (hCAII) and matrix metalloproteinase-2 (MMP-2).

Graphical Abstract



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Conflicts of interest

S.M.C. is a co-founder, has an equity interest, and receives income as member of the Scientific Advisory Board for Forge Therapeutics; is a co-founder, has an equity interest, and is a member of the Scientific Advisory Board for Blacksmith Medicines; and is a co-founder and has an equity interest Cleave Therapeutics (formerly Cleave Biosciences). These companies may potentially benefit from the research results of certain projects in the laboratory of S.M.C. The terms of this arrangement have been reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies.

Electronic Supplementary Information (ESI) available: Experimental details, Fig. S1–S9, Table S1–S4, CCDC 2211570-2211576, and PDB 8FAL and 8FAU contain the supplementary crystallographic data for this paper. See DOI: 10.1039/x0xx00000x

Thioamide-based compounds are described as a novel class of metal-binding pharmacophores (MBPs) for developing Zn(II)-dependent metalloenzyme inhibitors by masking thiol reactivity.

A critical role for metalloenzymes has been established in a wide range of human diseases including cancer, HIV/AIDS, hypertension, and fungal infection.[1] Inhibiting metalloenzymes can be achieved by binding molecules to the metal ions in their active sites. Fragment-based drug discovery (FBDD) is a powerful method to develop potent small molecule compounds for targeting metalloenzymes.[2] Starting from a weakly binding fragment, drug-like compounds can be obtained by utilizing strategies such as fragment linking or growth.[3] Several studies have described metal-binding pharmacophores (MBPs) for use as fragments for metalloenzyme inhibitor development.[4, 5]

Among the MBPs explored, thiol-based MBPs have been suggested as a useful class of fragments, especially for Zn(II)-dependent metalloenzymes.[6, 7] Captopril is a known inhibitor with a thiol ligand serving as an MBP for the Zn(II)-dependent angiotensin-converting enzyme (ACE).[8] The X-ray crystal structure of captopril bound to ACE reveals monodentate binding of the thiol functional group to the Zn(II) ion, which is further ligated by the protein residues His283, His387, and Glu411.[9] Unfortunately, captopril has adverse effects that are attributed to the thiol MBP, including rash and disruption of taste.[10, 11] Furthermore, the thiol moiety presents challenges for drug candidates more broadly because of the chemical reactivity and metabolic liability.[12, 13] Free thiols can be oxidized or form covalent adducts with biologically relevant thiol species such as cysteine residues or glutathione (GSH). This indiscriminate reactivity causes promiscuous biological activity that results in undesirable side effects.

To overcome some of these limitations, thioamides, thioureas, and thiocarbamates can serve as a thiol-like MBP with reduced liabilities. Thioamides, thioureas, and thiocarbamates are increasingly used as organosulfur ligands in coordination chemistry and are more stable when compared to other thiocarbonyls such as thioketones and thioaldehydes. Thioamides, thioureas, and thiocarbamates already plays a significant role in drug discovery. For example, propylthiouracil and methimazole are among the most important hyperthyroidism drugs used in the United States.[14] These drugs contain a thiocarbamide group that is essential for their hyperthyroidism activity.

In this work, thioamides, thioureas, and thiocarbamates compounds are proposed as MBPs for inhibiting Zn(II)-dependent metalloenzymes. The reactivity of these compounds with DTNB (5,5’-dithiobis(2-nitrobenzoic acid)) and cysteine derivatives was studied using UV-visible and NMR spectroscopy, as well as by HPLC as a proxy for their biostability. The biological activity of these novel MBPs was evaluated by performing inhibition assays against two Zn(II)-dependent metalloenzymes, human carbonic anhydrase II (hCAII) and matrix metalloproteinase-2 (MMP-2). In addition, their binding in a Zn(II) model complex was elucidated utilizing single crystal X-ray diffraction. Finally, the binding modes of these MBPs in the active site of hCAII were investigated using protein crystallography. The results show that these ligands are effective MBPs that can serve as useful fragments in future metalloenzyme fragment-based drug discovery (mFBDD) campaigns.

Eight (**1**-**8**, Fig. 1) were investigated as MBPs in this study. Compounds **1**, **4**, and **7** are thiocarbamates, compounds **2**, **3**, and **6** are thioureas, and compounds **5** and **8** are thioamides. Compounds **1**-**8** have been reported as bioactive compounds, but not widely explored as metalloenzyme inhibitors. Among them, **1** (benzo[d]oxazole-2-(3H)-thione) was reported as hCA inhibitor.[15] The metal-binding mode of **1** to the hCA active site was elucidated by protein crystallography and its activity against different hCA isoforms suggested the possibility of developing selective hCA inhibitors. Compounds **9** and **10** were also studied here, as they have been reported as thione-based MBPs for metalloenzyme inhibitors.[16] Lastly, **11**-**13** were studied as representative thiol-containing compounds.[6]

The reactivity of the compounds in Fig. 1 with DTNB (5,5'-dithio-bis-(2-nitrobenzoic acid)), also known as Ellman's reagent, was monitored spectroscopically (Fig. 2). DTNB reacts with sulfhydryl groups to produce a yellow-colored product, which can be quantified by its strong absorbance at 412 nm. Reactivity with DTNB was used as a surrogate for the propensity of the compounds to undergo disulfide exchange reactions with thiol-containing biomolecules and to qualitatively reflect the equilibrium of the tautomeric states between thione and thiol forms of each compound. Absorbance measurements were quantified against a standard calibration curve composed of known concentrations of L-cysteine methyl ester (**13**, Fig. S1). An excess of different DTNB ratios (1:2, 1:5, and 1:10) were used and the compounds and DTNB were incubated for 90 min prior to measuring the solution absorbance (Table S1).

Thiol compounds **11** and **12** showed essentially quantitative conversion with DTNB, as expected. Compound **9** showed ~50% conversion, while **10** was more variable (between 50 and 75 % with different DTNB ratios). The variability with **10** is tentatively attributed to the instability of the disulfide adduct between **10** and DTNB. Compounds **1**-**4**, **6**, and **8** showed essentially no reaction with DTNB after 90 min. Compounds **5** and **7** showed ~35% and ~20% conversion, respectively, which is significantly less than compounds **9**-**12**. The findings demonstrate that thioamide, thiourea, and thiocarbamate compounds are much less reactive with DTNB when compared with simple thiols. It also supports previous studies reporting that **1**-**8** predominantly exist as the thione tautomer,[17–23] which is important for bioactivity. The stability of the thione tautomer may prevent these compounds from being oxidized spontaneously to their corresponding disulfides.

As a secondary assay for biological stability, the reaction of compounds with L-cysteine methyl ester (**13**) was examined using HPLC to evaluable their stability with cysteine (Fig. S2). For this analysis, compounds were incubated with equimolar amounts of **13** for 24 h in 10% acetonitrile and 90% water at pH 7.4 (final concentration 1.25 mM). Compounds **1**-**7**, **9**, and **10** showed no reactivity with **13** under these conditions. By contrast, compounds **8** and **11** were found to generate disulfide products with **13** as revealed by the emergence of a new peak in the HPLC chromatogram. For **11**, several reaction products are observed, which are likely a combination of 2-mercapophenol-cysteine residues and products from the intrinsic oxidation of **11**. The findings observed in the HPLC analysis were confirmed by NMR analysis (Fig. S3).

Taken together, **1**, **2**, **3**, **4**, and **6** were found to be the most stable compounds in both the disulfide exchange reaction with DTNB and the disulfide formation reaction with a cysteine residue. The result implies that they can be considered stable fragments for inhibitor development. In addition, **5** and **7** are also promising as MBPs, as they have lower reactivity with DTNB and are stable in the presence of **13**.

The coordination chemistry of thioamides, thioureas, and thiocarbamates show monodentate, bidentate, and bridging modes. To elucidate the structural features of these MBPs, [$Tp^{Ph,Me}$Zn(**MBP**)] ($Tp^{Ph,Me}$ = hydrotris(5,3-methylphenylpyrazolyl)-borate) complexes were prepared as a bioinorganic model system. The [$Tp^{Ph,Me}$Zn(OH)] complex and its derivatives have been broadly used to mimic the tris(histidine) Zn(II) active site of many metalloenzymes (e.g., hCA or MMPs).[24, 25] The structures of the [$Tp^{Ph,Me}$Zn(**MBP**)] complexes of **1**, **2**, **3**, **4**, **5**, and **7** are shown in Fig. 3. Compounds **1**, **2**, **4**, **5**, and **7** coordinate to the Zn(II) center in a monodentate fashion via the sulfur atom while **3** coordinates to the Zn(II) center via the nitrogen atom. The C-S bond lengths of compounds **1**, **2**, **4**, and **7** in the [$Tp^{Ph,Me}$Zn(**MBP**)] complexes are in the range of 1.72 – 1.73 Å, which are typical bond lengths of C-S partial double bonds (Table S2). The coordination behavior suggests the propensity of the electrons to delocalize toward the sulfur atom upon deprotonation when in proximity to the Zn(II) center. Notably, compound **5** has the longest C-S bond length (1.77 Å), which is almost identical to **11**, which contains a C-S single bond (Fig. S4, Table S2). Interestingly, deprotonation of **5** at the C3-position was observed in the [$Tp^{Ph,Me}$Zn(**5**)] with concomitant C-C bond shortening. To confirm this unusual coordination mode, this deprotonation was confirmed with **5** bound to another Zn(II) model complex (see ESI for details, Fig. S5). This finding indicates that the proton at the C3-position in **5** has a lower pK_a when compared to the amine proton in this ligand. Previous reports indicate **5** is in an equilibrium between the 2-indolinethione and the 2-mercapto-indole, with the 2-indoleinethione predominant in solution.[22] The observations with the two different model complexes here confirmed the dominant equilibrium state of **5** and also reveal its coordination behavior at these biomimetic Zn(II) centers.

To evaluate these MBPs as potential fragments for Zn(II) dependent metalloenzyme inhibitors, hCAII and MMP-2 were selected as representative Zn-dependent metalloenzymes (Table 1, Fig. S6, S7). Both MMP-2 and hCAII contain a catalytic Zn(II) ion coordinated by three histidine residues and a water molecule in a tetrahedral geometry.[26, 27] Compounds **1** (K_i = 0.97 μM)[15] and **11** (K_i = 0.63 μM)[6] are reported inhibitors of hCAII. Compounds **9** and **10** are reported inhibitors for MMP-2 (IC_{50} = 60 and 140 μM, respectively).[16]

The screening results show that **4** and **5** have significant inhibition against both hCAII and MMP-2. It was interesting that **7**, which shares the same thiazole scaffold as **4** but contains a phenyl group, showed 15-fold better activity against hCAII compared to **4** but poorer performance against MMP-2. Compound **5**, which showed the best IC_{50} value for both enzymes, was further investigated. *N*-Methylated (**5a**) and *C*-dimethylated (at C3-position, **5b**) derivatives of **5** were prepared to see the effect of thione/thiol tautomerism on the inhibition activity. Both compounds exhibited a decrease in the inhibition activity, with **5b** showing a more significant loss of inhibitory activity. This result confirms the importance of

C3-deprotonation for metal ion coordination by these ligands. It is worth noting that despite the previous report of **1** as a hCAII inhibitor,[15] compound **1** was not active under the assay conditions used in the present study (which are not the same as the prior report).

To elucidate the binding mode of the hit compounds, the structure of the adducts formed between **4** and **7** with hCAII was determined by X-ray crystallography. Crystals of the protein complexes were obtained by soaking crystals of ~50–150 microns in diameter in 8 mM solutions of the compounds for 2–3 days (see ESI for details, Table S4).

The structure of **4** bound to hCAII shows a monodentate coordination through the exocyclic sulfur atom (Fig. 4, Fig. S8). A water molecule is also bound to the Zn(II) center, changing the coordination geometry from tetrahedral to a distorted trigonal bipyramidal (Fig. 4a). The same distorted trigonal bipyramidal coordination geometry was observed in the structure of **1** bound to hCAII (PDB:6YQU).[15] Both structures engage in hydrogen bonding with the Zn-coordinated water molecule through an oxygen atom for **1** and a nitrogen atom for **4**. The Zn-S distance of **4** is 2.63 Å, which is a shorter than the reported corresponding distance of **1** (2.75 Å),[15] and both are within the range of pentacoordinate Zn(II) complexes with different sulfur donor ligands (2.25 to 2.93 Å).[28, 29]

Compound **7** binds via monodentate coordination through the exocyclic sulfur atom to the Zn(II) center in tetrahedral geometry (Fig. 4, Fig. S8). Unlike **1** and **4**, no Zn-bound water molecule is observed in the structure of **7**. However, a water-mediated hydrogen bond interaction was observed through the water molecule positioned between the nitrogen atom of **7** and Thr200. The thiazole moieties both of **4** and **7** have the same binding orientation but the phenyl group is rotated in **7**, showing flexibility to occupy the active site (Fig. S8). The phenyl ring of **7** could be derivatized to establish further interactions with additional regions of the hCAII active site. Note that the Zn-S distance of **7** (2.30 Å) is shorter than that observed for compounds **1** and **4** (2.75 Å and 2.63 Å, respectively), suggesting strong metal-ligand interactions.

The binding modes of compounds **4** and **7** were compared with the known thiol-based inhibitor **11** (PDB: 2OSM) bound to hCAII (Fig. S9). When comparing the binding conformations, **7** has a binding geometry that more closely resembles **11** than **4**. The thiazole ring of **7** and the phenol ring of **11** are well aligned in the same plane and display angles and distances in a similar range. Furthermore, there is a clear difference in the binding conformation of these compounds when compared to a canonical benzenesulfonamide inhibitor of hCAII (PDB: 2WEJ, Fig. S9). While the binding angle and distance of the nitrogen in benzenesulfonamide to the Zn center is 1.95 Å and 120°, sulfur-containing inhibitors **4** and **7** have longer distances (2.30–2.63 Å) and more shallow angles (102–105°).

In conclusion, several thioamide, thiourea, and thiocarbamate MBPs that are less reactive than free thiols, but still have enzymatic inhibition activity against Zn(II)-dependent metalloenzymes, including hCAII and MMP-2, have been identified. The Zn-S binding interaction of these compounds was investigated using bioinorganic model complexes and hCAII. Compounds **5** and **7** showed higher stability and better activity against hCAII

compared to the known thiol-based inhibitor **11**. Given the selectivity of **7** toward hCAII over MMP-2 and the synthetic handle provided by the phenyl group, **7** can be an attractive starting point for developing new hCAII inhibitors. Based on the stability and comparable potency for hCAII and MMP-2, compound **4** may be among the more promising new MBPs from this study. This work demonstrates the potential utility of thioamide, thiourea, and thiocarbamate MBPs as fragments for mFBDD campaigns.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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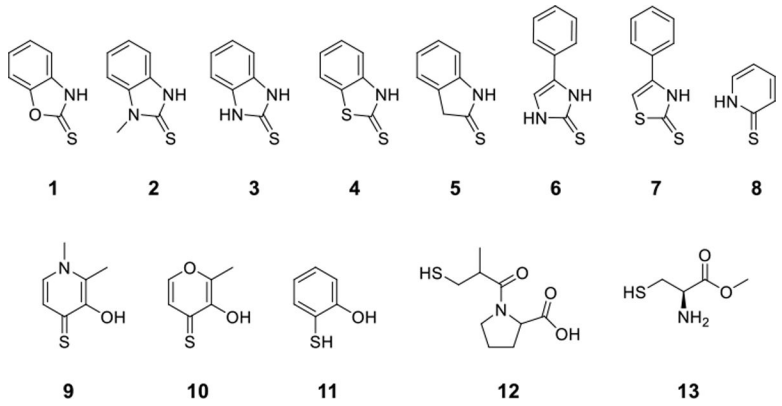

Fig. 1.

Thioamide, thiourea, and thiocarbamate MBPs proposed for use in Zn(II)-dependent metalloenzymes. Compounds **9** and **10** were utilized as known thione-based MBPs and 2-mercaptophenol (**11**), captopril (**12**), and L-cysteine methyl ester (**13**) were used as representative thiol-based compounds in this paper.

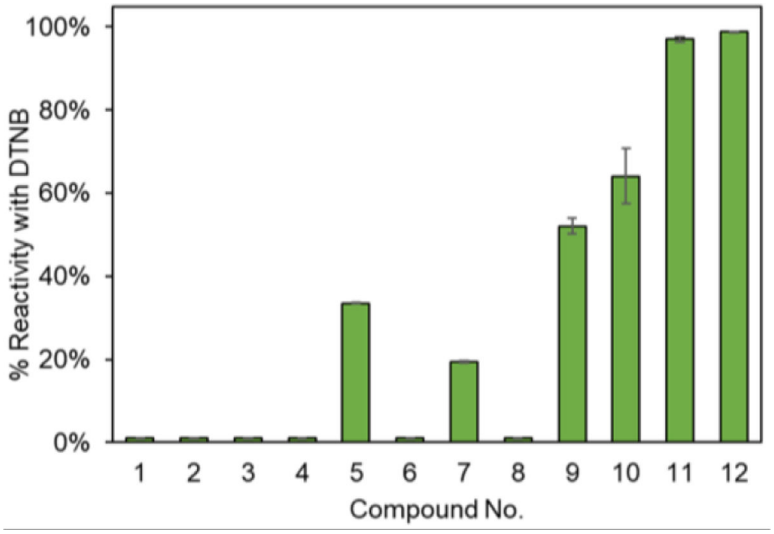

Fig. 2.

Percent reactivity of compounds **1-12** with DTNB. The molar ratio of compounds tested to DTNB was 1:10 with an incubation time of 90 min. 2-Mercapophenol (**11**) and captopril (**12**) were used as representative thiol-based compounds that showed essential quantitative disulfide formation with DTNB.

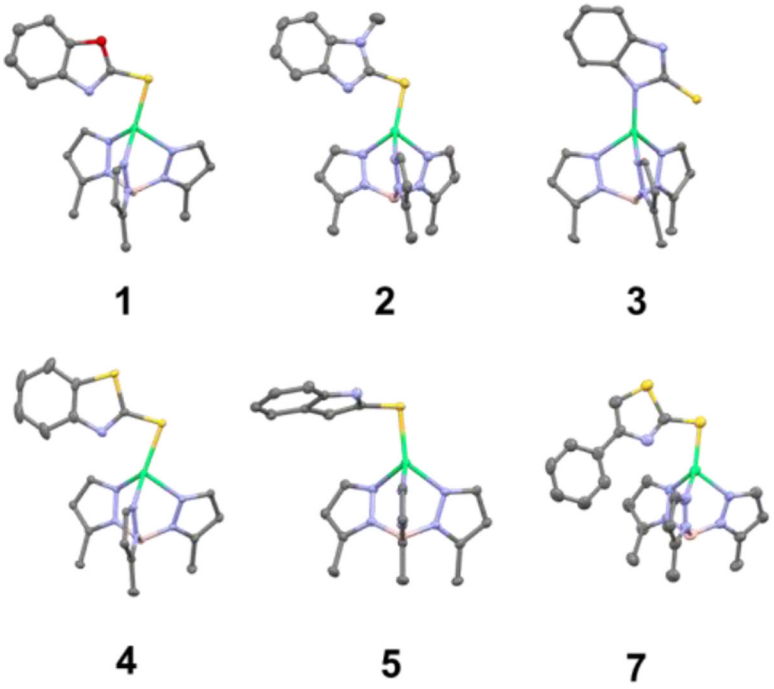

Fig. 3.

Crystal structure of [$Tp^{Ph,Me}$Zn(**MBP**)] complexes (ORTEP, 50% probability ellipsoids). Hydrogen atoms and phenyl groups from the $Tp^{Ph,Me}$ ligand were removed for clarity. Color scheme: carbon = gray, nitrogen = blue, oxygen = red, sulfur = yellow, boron = pink, and zinc = green.

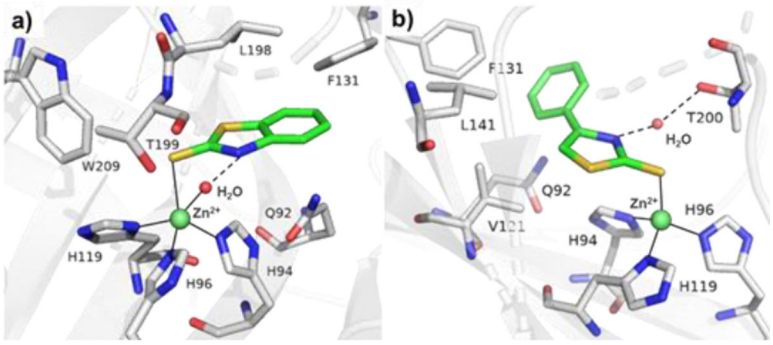

Fig. 4.

Structure of a) **4** (PDB: 8FAL) and b) **7** (PDB: 8FAU) bound to hCAII. Zn(II) coordination is represented by solid lines and hydrogen bonding is represented by dashed lines. Zn(II) ion and water molecules are shown as green and red spheres, respectively. Atom colors are: carbon (green for MBP, gray for protein), oxygen (red), nitrogen (blue), and sulfur (yellow).

Table 1.

IC_{50} values of the proposed compounds against hCAII and MMP-2. IC_{50} values reported in μM with the 95% confidence interval indicated. IC_{50} titration curves can be found in the ESI (Fig. S6 and S7).

Compound	IC_{50} (μM)	
	hCAII	MMP-2
1	>200	>200
2	>200	>200
3	>200	>200
4	68.27 ± 10.69	102.2 ± 12.7
5	3.42 ± 0.34	76.64 ± 4.26
5a	20.34 ± 2.09	103.4 ± 10.6
5b	>200	>200
6	>200	>200
7	4.56 ± 0.62	>200
8	>200	>200
9	>200	25.85 ± 1.08
10	>200	44.52 ± 3.01
11	19.40 ± 5.15	>200