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Metal-mediated inhibition is a viable approach for inhibiting cellular methionine aminopeptidase

Sergio C. Chai, Qi-Zhuang Ye *

Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN 46202, USA

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ABSTRACT

Methionine aminopeptidase (MetAP) plays an essential role for cell survival. Hence, MetAP is a promising target for developing broad spectrum antibacterial agents. MetAP can be activated in vitro by a number of divalent metals, and X-ray structures show that the active site can accommodate two cations. Herein, we demonstrate bacterial growth inhibition by a compound that targets MetAP by recruitment of a third auxiliary metal. Contrary to previous beliefs, this shows that metal-mediated inhibition is a viable approach for discovering MetAP inhibitors that are effective for therapeutic application.

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Methionine aminopeptidase (MetAP) is a ubiquitous enzyme that cotranslationally removes the initiator methionine residue from newly synthesized proteins.[1] This is an essential process for cell survival, because deletion of MetAP gene was shown to be lethal in a number of microorganisms, including *Escherichia coli*,[2] *Salmonella typhimurium*,[3] and *Saccharomyces cerevisiae*.[4] Most bacteria have only a single MetAP gene, and therefore, it is a promising target for developing antibiotics with a novel mechanism of action and broad antibacterial spectrum. This enzyme as a purified protein requires divalent cations, including Fe(II), Mn(II), Co(II), Ni(II) and Zn(II), for catalytic activity.[5] Based on X-ray structures, most MetAP inhibitors interact directly with the two metal cofactors at the dinuclear active site,[6] and some recruit a third metal to form a trimetalated enzyme-inhibitor complex (Fig. 1A).[7–10] The inhibitors that rely on the trimetalated form for inhibition are generally discounted, because the cellular concentration of metal ions is believed not high enough to form such enzyme-inhibitor complexes.[10] However, now we report the observation of antibacterial activity of one of such inhibitors (**1** in Fig. 1B) through cellular MetAP inhibition. This indicates that metal-mediated inhibition can be successful in designing effective inhibitors for MetAP and possibly for other enzymes as well.

The active site of MetAP is a shallow and mostly hydrophobic pocket with two metal ions (M1 and M2) sitting at the bottom, coordinated by five conserved residues.[6] We reported that inhibitor **1** binds to *E. coli* MetAP through the third metal ion (M3) to a conserved histidine (H79) and three water molecules (w1, w2 and w3), forming an octahedral arrangement.[8] The affinities of the first and the second metals to *E. coli* MetAP are significantly different. Co(II) or Mn(II) binds at the first site with K_d of 0.2 or 6 μM, respectively, while Co(II) binds at the second site with K_d of 2.5 mM.[11,12] The affinity to the third metal site must be even weaker, because the metal has the only contact to the enzyme through

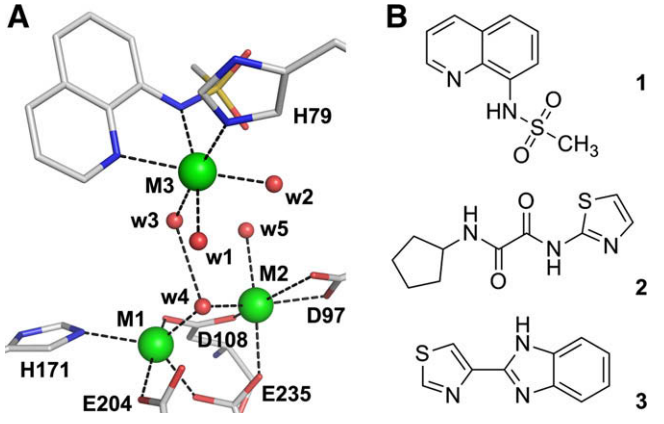

Figure 1. MetAP inhibition through a trimetalated enzyme-inhibitor complex. (**A**) Trimetalated site with inhibitor **1** bound (carbon gray, nitrogen blue, oxygen red, sulfur yellow). Metal ions are shown as green spheres (M1–M3), and water molecules that directly coordinate with metal are shown as red spheres (w1–w5). (**B**) Structures of the inhibitors **1**–**3**.

* Corresponding author. Tel.: +1 317 278 0304; fax: +1 317 274 4686.
E-mail address: yeq@iupui.edu (Q.-Z. Ye).

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an imidazole nitrogen of H79, and such a trimetalated X-ray structure has never been obtained in the absence of an auxiliary ligand. Although these inhibitors display submicromolar potency (IC_{50}, 0.14 μM for **1**,[8] 0.067 μM for **2**;[13] and K_i, 0.4 μM for **3**[10]), this high potency is attributed to the high metal concentrations used during in vitro assays. These types of inhibitors were not recommended as lead compounds for drug discovery, because they were not expected to function in living cells because of limited amount of metals.[10]

Surprisingly, inhibitor **1** prevented the growth of *E. coli* cells. Three *E. coli* strains (AS19, D22 and SM101) were used, and **1** inhibited the growth at IC_{50} of 38 μM, 54 μM and 113 μM, respectively. However, inhibitor **2**, with the same binding mode,[14] failed to halt the cell growth up to 1 mM, the highest concentration tested. MetAP carries out an essential function in *E. coli*,[2] and MetAP inhibition leads to inhibition of cell growth.[15] To confirm the observed growth inhibition is caused by MetAP inhibition, we monitored the N-terminal processing of recombinant glutathione-S-transferase (GST) of chicken liver as the biomarker in *E. coli* cells.[15] The GST protein was purified by affinity chromatography and analyzed by mass spectrometry, revealing masses for processed GST at 26,114 and unprocessed GST at 26,245 (Fig. 2). Only processed GST was detected in the absence of **1**. In contrast, significant amount of unprocessed GST was evident in cells incubated at a sub-lethal concentration of **1**. These results provide the evidence that the cell growth inhibition by **1** is due to the effective inhibition of cellular MetAP enzyme. Consistent with our observation is the report that pyridine-2-carboxylic acid derivatives were shown to arrest growth in tumor cell lines by inhibiting human type 1 MetAP, and those inhibitors also require the third metal for inhibition.[16]

There is ample evidence that transition metals are tightly regulated in cells, with very small amounts of cations bioavailable in solution.[17] To form a trimetalated enzyme-inhibitor complex, the inhibitor may acquire the metal through competitive binding from other complexes. Another possibility is to use a more abundant metal to fill the third metal site. To test the formation of hybrid trimetalated complexes with **1**, *E. coli* MetAP in apoform was activated by Mn(II) (10 μM). Supplementation with extra metal (40 μM of Co(II), Mn(II), or Fe(II); 100 μM of Ca(II); or 1 mM of Mg(II)) will provide additional cations that assist in formation of the complex. Comparing with the sample with no extra metal added, all of the cations enhanced the inhibition potency, with the most dramatic effect from Co(II), followed by Fe(II) and Mn(II) (Fig. 3). Ca(II) and Mg(II) are not MetAP activators but are relatively

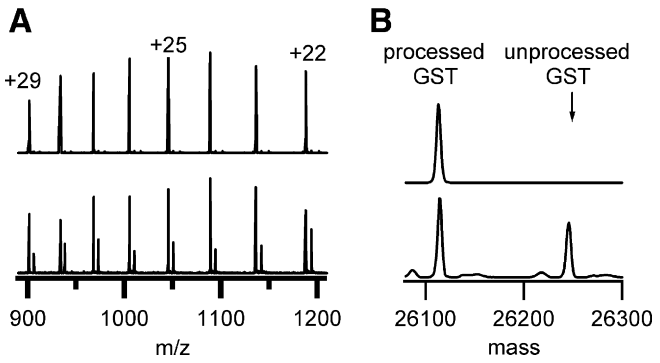

Figure 2. Analysis of N-terminal processing of recombinant GST protein by mass spectrometry. (**A**) ESI-MS protonation multiplicity spectra of a mixture of processed and unprocessed GST with charge states ranging from +22 to +29. The spectrum corresponding to the sample in the absence of **1** is shown on top, and the spectrum representing the sample in the presence of **1** is displayed at the bottom. (**B**) The spectra from A were transformed to a mass scale. The mass difference (131) between both peaks corresponds to a methionyl residue.

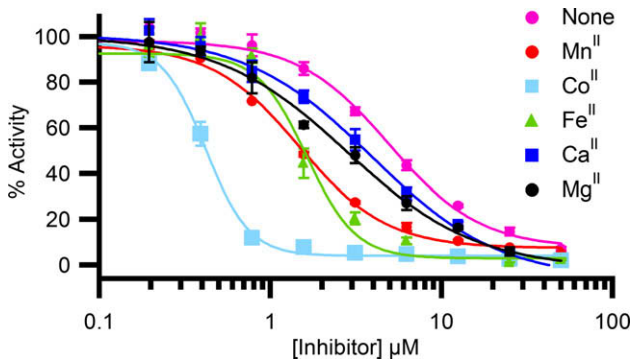

Figure 3. Enhancement of inhibition of *E. coli* MetAP by **1** upon addition of metal ions. Apo-MetAP was activated by Mn(II), and the enzymatic activity was monitored at various concentrations of **1** in the presence of the metal ions.

abundant in cells (Ca(II), 0.1 mM; Mg(II), >10 mM).[17] Their enhancement is moderate but noticeable at the concentrations tested. These results suggest that **1** can inhibit MetAP activity in vivo by recruiting an auxiliary metal from a variety of candidate metals. Consequently, the requirement of high levels of a specific metal to form the ternary complex under assay conditions can in principle be compensated by the collective concentrations of different types of metals under physiological conditions. The use of mixed-metals in inhibition is reminiscent to the activation of metalloenzymes by hybrid metals.[18]

It is puzzling that **1** inhibited cell growth effectively but **2** did not. We reported previously that **2** is highly selective for different metalloforms (IC_{50}, Co(II), 0.067 μM; Mn(II), 53 μM; Ni(II), 1.0 μM; Fe(II), 46 μM),[13] while **1** is much less so (IC_{50}; Co(II), 0.137 μM; Mn(II), 2.14 μM; Ni(II), 0.184 μM; Fe(II), 3.74 μM).[8] Although **1**–**3** are all bidentate ligands for the metal, **1** binds differently with a rotational offset from **2** and **3** when the three X-ray structures are overlaid (Fig. 4). The angle formed with the metal is much smaller for **1** (76.0°) than for **2** (79.7°) or **3** (81.3°), and the distance to the metal is also much longer for **1** (2.22, 2.32 Å) than for **2** (2.13, 2.21 Å) or **3** (2.07, 2.22 Å). The differences may be the structural basis for the relaxed metalloform selectivity of **1**, in comparison with **2** and **3**, and we propose that the relaxed metal selectivity is the key for its ability of recruiting different metals to the third metal site.

These metal-mediated inhibitors **1**–**3** were somewhat mistakenly identified by screening assays with high metal concentrations and possess high inhibitory potency. It was reported that Zn(II) at 100 nM enhanced the potency of a serine protease inhibitor by 3800-fold to a K_i of 5 nM, through Zn(II) coordination with two residues (His and Ser).[19] In our case, the inhibition of **1** is mediated by a metal ion interacting with only one His residue, assisted by an extended hydrogen bond network in the active site pocket (Fig. 1A),[8] and it appears that several different metal ions, including those with high abundance, can function as the mediator. The active site pocket of MetAP is lined with conserved and non-conserved residues for potential metal ligation, and conceivably, potential metal binding sites exist in the active site of other enzymes or pockets of many structural or functional proteins. High throughput screening is a powerful technology in identifying compounds with unique biophysical properties, such as **1** and the metalloform-selective MetAP inhibitors we reported previously.[13,20] By adjusting the screening condition with a low metal concentration, we also discovered a MetAP inhibitor with specificity for a monometalated enzyme.[21] The demonstration of antibacterial activity of **1** through inhibition of cellular MetAP enzyme shows that metal-mediated inhibition is a viable approach for discovering MetAP inhibitors that are effective for therapeutic

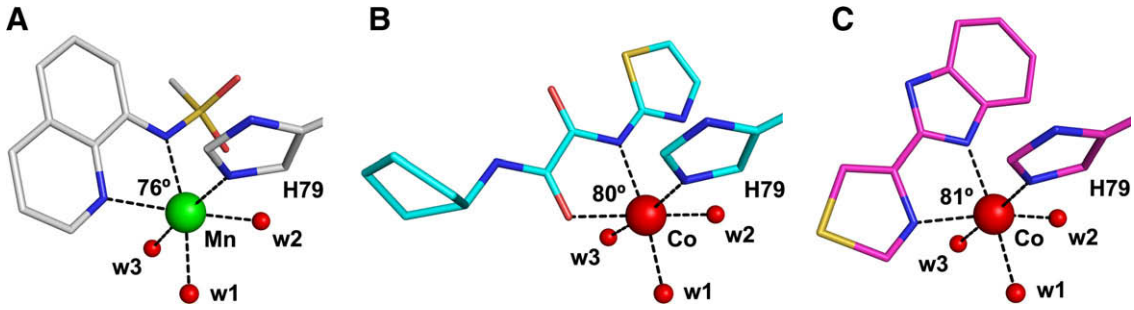

Figure 4. Differences in binding of inhibitors at the metal M3 site. The metal site is formed by the bidentate ligand **1** (**A**, carbon gray, nitrogen blue, oxygen red, sulfur yellow), **2** (**B**, carbon cyan) or **3** (**C**, carbon magenta) through the conserved H79 of MetAP protein. Three water molecules are shown as small red spheres, and the metal ion is shown as large spheres (Mn green and Co red). Black dash lines show the octahedral coordination. The angles formed from the two coordinating atoms of the bidentate ligand to the metal ion are indicated.

application, and it also calls for exploring the potential metal sites on protein surface for drug discovery and development.

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Supplementary data

Experimental procedures for inhibition of purified enzyme, inhibition of cell growth, and analysis of the biomarker GST by MS are reported. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.10.082.

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