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Growth inhibition of *Escherichia coli* and methicillin-resistant *Staphylococcus aureus* by targeting cellular methionine aminopeptidase

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Abstract

Methionine aminopeptidase (MetAP) catalyzes the N-terminal methionine excision from the majority of newly synthesized proteins, which is an essential cotranslational process required for cell survival. As such, MetAP has become an appealing target for the development of antimicrobial therapeutics with novel mechanisms of action. By screening a library of small organic molecules, we previously discovered a class of compounds that selectively inhibit the Fe(II)-form of MetAP. Herein, we demonstrate that some of these compounds and their newly synthesized derivatives halt the growth of *Escherichia coli* and *Staphylococcus aureus* cells with significant potency. The most potent compound inhibited methicillin-resistant *S. aureus* (MRSA) growth with an IC₅₀ value of 1 μM and MIC of 0.7 μg/ml. Two cell-based assays were used to verify that MetAP is the intracellular target in *E. coli* cells. These findings can serve as foundation for the development of novel therapeutics against an ever increasing threat by drug resistant staphylococcal infections.

Keywords

antibiotic; drug resistance; drug discovery; enzyme inhibition; hydrolase; metalloenzyme

1. Introduction

Methionine aminopeptidase (MetAP) is a metalloenzyme involved in the critical protein maturation by catalyzing removal of the N-terminal initiator methionine from nascent proteins [1, 2]. MetAP is a ubiquitous enzyme found in all prokaryotes and eukaryotes, and the lethal effect of MetAP gene deletion has been reported for *Escherichia coli* [3], *Salmonella typhimurium* [4], and *Saccharomyces cerevisiae* [5]. Therefore, MetAP is an appealing target for the development of broad-spectrum antibacterial and antifungal drugs with a novel mechanism of action [6]. Morphological changes in *E. coli* cells were observed when expression of MetAP gene was attenuated by genetic regulation [7, 8] and when the

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cellular MetAP activity was inhibited by using an inhibitor [7]. However, it is puzzling that even though an extensive array of small molecules has been reported to inhibit the purified MetAP enzyme with high potency, almost all of them failed to show any significant antibacterial activity [9–11].

Hydrolysis of proteins and peptides by MetAP is accomplished with the assistance of divalent metal ions that serve as a cofactor for the catalytic reaction [12]. MetAP in the apoenzyme form can be reproducibly activated by a number of divalent metal ions, including Co(II), Mn(II), Ni(II), and Fe(II) [13, 14]. According to X-ray structures, the catalytic site is a shallow pocket with two metal ions, usually Co(II) or Mn(II), situated at the bottom, forming a dinuclear arrangement [15]. Because Co(II) is one of the best activators and several MetAPs were initially identified as Co(II) enzymes [15], most of the current MetAP inhibitors were discovered and characterized by using a MetAP enzyme in the Co(II)-form. We demonstrated that potent inhibitors of one metalloform may not inhibit the same MetAP that is activated by another metalloform [13, 16]. There is the possibility that the metal cofactor utilized in reconstituting the purified MetAP for inhibitor screening is not physiologically relevant, resulting in compounds that cannot inhibit the cellular MetAP. When MetAP was overexpressed in *E. coli*, the intracellular amount of Fe(II) was doubled, while Co(II) levels remained undetectable compared to samples without MetAP overexpression [14]. Therefore, D'Souza et al. [14] suggested that *E. coli* MetAP functions as a Fe(II) enzyme. We have developed several sets of metalloform-selective MetAP inhibitors based on high throughput screening hits [16, 17], and these inhibitors can target either the Co(II)-, Mn(II)- or Fe(II)-form of *E. coli* MetAP with both high potency and selectivity. We used these metalloform-selective inhibitors to characterize inhibition of purified *E. coli* MetAP *in vitro*, inhibition of the same enzyme residing in intact *E. coli* cells, and inhibition of bacterial cell growth [18]. The fact that only the Fe(II)-form selective inhibitors showed antibacterial activity on several *E. coli* and *Bacillus* strains led us to conclude that Fe(II) is the likely metal used by MetAP in *E. coli* and *Bacillus* cells [18].

All of the Fe(II)-form selective MetAP inhibitors have a catechol moiety (Fig. 1). We have solved an X-ray structure of *E. coli* MetAP in complex with one of the inhibitors, demonstrating that they interact directly with the metal cofactor at the MetAP enzyme active site through coordination by the catechol hydroxyl groups [17]. Based on their initial antibacterial activity on *E. coli* and *Bacillus* cells, we evaluated some of these inhibitors for growth inhibition against a drug-susceptible *Staphylococcus aureus* strain, and a methicillin-resistant *S. aureus* (MRSA) strain. In addition, we used two cellular MetAP activity assays to confirm that their antibacterial activity was a result of targeting cellular MetAP, validating the notion that MetAP is a promising drug target for the development of novel antibacterial therapeutics.

2. Results and discussion

2.1 Growth inhibition of *E. coli* and *S. aureus* strains by the Fe(II)-form selective MetAP inhibitors

Previously, we evaluated some of the compounds against several *E. coli* and *Bacillus* strains, and they displayed considerable antibacterial activity [17–19]. In general, we noticed that the Gram-positive *Bacillus* strains were more sensitive than the Gram-negative *E. coli* strains. We envisioned that these compounds could have the same effect on other Gram-positive strains. With increasing threats from antibiotic resistant staphylococcal infections, we decided to test these compounds for growth inhibition of *S. aureus* ATCC 43300 strain (susceptible) and *S. aureus* ATCC BAA1680 strain (methicillin-resistant) (Table 1). Using the growth inhibition of *E. coli* AS19 strain as a guide, we selected compounds **1–7** and evaluated them on the two *S. aureus* strains. All of them showed considerable activity, and it

is also interesting to note that the growth inhibition for the three strains tested is parallel. This is not surprising because all bacterial MetAPs, including the ones from *E. coli* and *S. aureus*, belong to the type 1 MetAP with high sequence homology. Different *S. aureus* strains, such as Newman [20], N315 and M50 [21], have the same MetAP sequence. Therefore, it is conceivable that a MetAP inhibitor will affect the cell growth of susceptible and methicillin-resistant strains equally well. Compound **2** was the best with IC₅₀ at 2.8 μ M or MIC at 1.0 μ g/ml for the susceptible *S. aureus* strain and IC₅₀ at 1.0 μ M or MIC at 0.7 μ g/ml for the MRSA strain. Similarly, compound **7** showed potent inhibition of the MRSA strain, as well as the susceptible *S. aureus* strain and *E. coli* strain. Structurally, both **2** and **7** were polychlorinated.

2.2 Inhibition of the cellular MetAP activity by these MetAP inhibitors by monitoring the hydrolysis of a synthetic substrate in live *E. coli* cells

Cell-based assays are powerful tools in target identification and confirmation. In order to verify that the cellular target of these inhibitors is MetAP, we decided to utilize two cell-based assays that we have developed recently [18]. One of the assays is based on overexpressing *E. coli* MetAP in *E. coli* cells and making these cells permeable to substrates and inhibitors by Ca(II) treatment [18]. With the enzymatically active MetAP residing in live *E. coli* cells, we can monitor the hydrolysis of the fluorogenic substrate methionyl aminomethylcoumarin (Met-AMC) by the cellular MetAP by fluorescence. We previously found that the rate of Met-AMC hydrolysis reached maximum between 200 μ M and 10 mM of CaCl₂ [18], indicating that this range of Ca(II) ions is optimal for substrate penetration into the cells. Therefore, we chose 5 mM as the Ca(II) concentration for our cellular MetAP activity assay. The Ca(II)-treated cells were incubated with the compounds at different concentrations, and the calculated IC₅₀ values are reported in Table 1. Many of the compounds displayed considerable inhibition of intracellular Met-AMC hydrolysis, indicating a concomitant inhibition of intracellular MetAP. Compounds **1** and **2** were among the most efficient inhibitors in this cellular assay, with IC₅₀ values of 15.4 and 22.1 μ M respectively. We noticed that the IC₅₀ values obtained by this cellular assay were generally larger than those obtained by the cell growth assays. One possibility is that in the cellular assay, the recombinant MetAP was present at a higher concentration. Another reason is that a higher cell density was used in the cellular MetAP activity assay compared to the cell growth assay. Difference in cell penetration by these inhibitors could also affect their observed antibacterial activity.

2.3 Inhibition of the cellular MetAP activity by these MetAP inhibitors by monitoring the N-terminal processing of a recombinant GST protein in *E. coli*

The second cell-based assay to validate the intracellular target is based on monitoring the presence of the unprocessed GST that retains the N-terminal methionine as an indication of MetAP inhibition in cells. The N-terminal methionine residue of chicken-liver GST was efficiently removed when the protein was expressed in *E. coli* [22]. Cells harboring the GST-expressing plasmid were cultured at sub-lethal concentrations of the inhibitors **1** and **7**, and bacterial cell growth was retarded with the concomitant accumulation of unprocessed GST protein. Inhibitors **1** and **7** were chosen for the testing because of their potency and availability and their convenience to achieve sub-lethal inhibition. Both processed and unprocessed proteins were purified from cell extracts by affinity chromatography and subsequently analyzed by mass spectrometry (Fig. 2). The spectra obtained from the ESI-MS for cells incubated in the absence or presence of inhibitors are illustrated in Fig 2A. The deconvoluted spectra (Fig.2B) showed two peaks with masses 26,114, corresponding to the processed GST, and 26,245, corresponding to the unprocessed GST (Fig. 2B, top and middle spectra). The mass difference of 131 correlates with a mass shift that corresponds to a methionyl residue. In the absence of the inhibitors, almost all of GST was processed, and

only the protein lacking the initiator methionine was detected (Fig. 2B, bottom spectra). Glutathione used in the elution of GST from the affinity column was removed from the sample by buffer exchange. However, small quantities of the glutathione-bound GST (processed and unprocessed forms) could be sometimes observed in the ESI-MS spectra (Fig. 2A, middle spectra).

3. Conclusion

Infectious diseases remain one of the largest culprits of mortality in the world [23]. It was estimated that in the United States alone, bacterial infections account for 90,000 deaths a year [24]. Drug-resistant pathogenic bacterial strains are hard to treat with conventional therapies. MRSA, in particular, has become a serious threat, with 100,000 people seriously affected per year and an estimated 19,000 related deaths [24]. Therefore, there is a great need to develop therapeutics with novel mechanisms of action. MetAP has been demonstrated to be an essential enzyme for cell survival in a number of organisms, including bacteria. To our knowledge, the inhibitors that we report herein represent the first MetAP inhibitors that significantly prevent growth of MRSA, validating MetAP as a promising antibacterial target and serving as foundation for the discovery of more effective antibacterial therapeutics.

4. Experimental

4.1 Materials

The bacterial strains, the susceptible *S. aureus* ATCC 43300 and the methicillin-resistant *S. aureus* ATCC BAA1680 [25], were acquired from the American Type Culture Collection (Manassas, VA). The *E. coli* AS19 strain was obtained as a gift from Prof. Liam Good at Karolinska Institute. This strain has a severely depleted lipopolysaccharide layer, but its exact mutation is unknown [26]. Mueller Hinton broth and agar were purchased from Remel Products (Lenexa, KS). The syntheses of inhibitors **3–7** were described [17]. Inhibitors **1** and **2** were similarly synthesized in our laboratory and characterized as the following. Inhibitor **1**, ^1H NMR (DMSO- d_6 , 500 MHz) δ 9.30 (s, 1H), 9.26 (s, 1H), 7.57 (d, J = 5.5 Hz, 1H), 7.09 (d, J = 5.5 Hz, 1H), 7.05 (d, J = 2.5 Hz, 1H), 6.91 (dd, J = 8 Hz, 2.5 Hz, 1H), 6.81 (d, J = 8 Hz); ^{13}C NMR (CD_3OD , 125 MHz) δ 147.00, 146.47, 137.99, 130.13, 125.35, 124.29, 121.73, 121.22, 116.93, 116.58. Inhibitor **2**, ^1H NMR (CD_3OD , 500 MHz) δ 7.07 (s, 1H), 6.94 (d, J = 8.0 Hz, 1H), 6.83 (d, J = 8.0 Hz, 1H); ^{13}C NMR (CD_3OD , 125 MHz) δ 146.61, 145.36, 135.30, 123.27, 122.15, 121.55, 120.08, 117.67, 115.26, 115.01. The fluorogenic substrate, Met-AMC, was purchased from Bachem Bioscience (King of Prussia, PA). Tris(hydroxymethyl) aminomethane (Tris), CaCl_2 , monobasic and dibasic sodium phosphate, ascorbic acid, glutathione, dithiothreitol, HPLC-grade methanol, acetic acid, LB media, ampicillin, and all ingredients necessary to prepare the MOPS buffer [27] for GST induction were acquired from Fisher Scientific (Pittsburgh, PA). The pBACE vector with the cGSTA1 insert [22] was generously provided by Prof. Ming F. Tam at Institute of Molecular Biology, Academia Sinica, Taiwan.

4.2 Inhibition of bacterial growth

The assay was carried out on 384-well plates containing 12 dilutions of each inhibitor. Serial dilutions were performed using a Precision Microplate Pipetting System (BioTek, Winooski, VT). Bacterial cells grown to exponential phase at 0.5 McFarland optical density [28] was diluted 1000 fold in Mueller Hinton media, and the cell suspension was subsequently dispensed into the plates by Multidrop Combi (Thermo Scientific, Waltham, MA). Cell suspension (40 μl) was dispensed to 40 μl of inhibitor, and cell growth was monitored continuously by absorbance at 600 nm using a SpectraMax 340PC384 (Molecular Devices,

Sunnyvale, CA). Absorbance kinetic experiments were carried out for 10 h at 37 °C, with readings taken every 5 min. Signal intensities obtained from the growth curves at time points along the exponential phase corresponding to 50–85% of total intensity of an uninhibited sample were averaged and converted to percent inhibition to obtain IC₅₀ values, as previously described [18]. IC₅₀ and MIC are defined as the minimum compound concentration at which growth is inhibited by 50% and 90%, respectively.

4.3 Cellular MetAP activity assay using the fluorogenic substrate Met-AMC

BL21(DE3) cells overexpressing the recombinant *E. coli* MetAP were grown to exponential phase [18]. After harvesting and washing twice with deionized water, the cells were resuspended in 10 mM CaCl₂ in 100 mM Tris (pH 7.5) to allow for penetration of the fluorogenic substrate Met-AMC. Cell suspension (40 µl) was combined with 40 µl of various concentrations of inhibitor on a 384-well plate. Met-AMC hydrolysis to yield the fluorescent aminomethylcoumarin was monitored via fluorescence (λ_{ex} 360 nm, λ_{em} 460) at room temperature. The IC₅₀ values were calculated from the rate of substrate hydrolysis as described above.

4.4 Cellular MetAP activity assay using the recombinant glutathione S-transferase (GST) as a biomarker

E. coli AS19 cells were transformed with the pBACE expression vector encoding for chicken-liver GST A1-1 with a phenylalanine to alanine substitution at position 111 [22]. When cells reached exponential phase in LB medium containing 50 µg/ml ampicillin, the cells were diluted 10,000 fold into the induction MOPS medium described by Craig et al. [29]. The cells were allowed to grow at 25 °C for an additional 50 h in the presence or absence of inhibitors. Harvested cells were resuspended in PBS buffer containing 5 mM EDTA and lysed by French press. GST proteins were then purified by affinity chromatography using a GSTrap HP column (GE Healthcare, Piscataway, NJ). After washing the impurities out of the column with PBS buffer, the recombinant protein was eluted with 50 mM Tris-HCl containing 20 mM glutathione at pH 7.8. The purified protein was incubated with 5 mM dithiothreitol to remove protein-bound glutathione, and then the buffer was exchanged for deionized water by ultrafiltration. GST samples containing 50% methanol and 3% acetic acid was injected into an Agilent G1946B mass spectrometer interfaced with an electrospray ionization source (ESI-MS) in the positive mode. Deconvolution of the raw data to yield mass-based spectra was obtained with the software MagTran [30].

Research highlights

- Inhibitors of methionine aminopeptidase displayed antibacterial activity
- Growth of drug-susceptible and methicillin-resistant *S. aureus* was inhibited
- Cell-based assays validate methionine aminopeptidase as the intracellular target

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Figure 1.
Chemical structures of the MetAP inhibitors used in this study.

Figure 2. Effect of inhibition of MetAP on N-terminal processing of a recombinant GST protein. **A**, ESI-MS spectra of a mixture of processed and unprocessed GST for cells incubated in the presence of **7** (top) or **1** (middle). Cells grown in the absence of an inhibitor showed only the processed GST (bottom). **B**, the protonation multiplicity spectra from A were transformed to mass scale spectra.

Table 1

Inhibition of growth of *E. coli* AS19, *S. aureus* 43300 and *S. aureus* BAA1680 strains, and inhibition of the enzymatic activity of *E. coli* MetAP in *E. coli* cells

Compound	Growth Inhibition ^a			Inhibition of Cellular Activity of <i>E. coli</i> MetAP ^b
	<i>E. coli</i> AS19	<i>S. aureus</i> 43300	<i>S. aureus</i> BAA1680	
1	23.3 (5)	25.8 (12)	63.8 (25)	15.4
2	2.3 (0.7)	2.8 (1)	1.0 (0.7)	22.1
3	15.4 (4)	13.9 (16)	33.4 (11)	49.5
4	12.4 (5)	8.2 (5)	12.4 (4)	37.3
5	29.9 (15)	11.7 (11)	41.7 (13)	38.4
6	10.8 (5)	12.2 (18)	23.2 (13)	63.6
7	6.1 (3)	11.3 (4)	4.6 (2)	51.9

^aIC₅₀ values (μM) are reported along with MIC values (μg/mL) at 90% inhibition as shown in parenthesis.

^bIC₅₀ values in μM.