Amino Acid Thioesters Exhibit Inhibitory Activity against B1–B3 Subclasses of Metallo-β-lactamases

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Superbug infection caused by metallo-β-lactamases (MβLs) is a global public health threat. Previous studies reported that the thioesters specifically inhibited the B3 subclass MβL L1. In this work, nine amino acid thioesters 1–9 were synthesized, the activity evaluation revealed that all of these molecules exhibited broad-spectrum inhibitory efficacy against ImiS, IMP-1, NDM-1, and L1, with IC₅₀ values range of 0.02–54.9 µM (except 5 and 7 on NDM-1), and 1 was found to be the best inhibitor with IC₅₀ range of 0.02–16.63 µM. Minimal inhibitory concentration (MIC) assays showed that thioesters 1, 5 and 9 restored 2–32-fold antibacterial activity of cefazolin and/or imipenem against both Escherichia coli BL21 and DH10B strain expressing ImiS, L1, IMP-1 and NDM-1 (except 5 on NDM-1), and also, thioester 1 increased 2–4-fold antimicrobial activity of cefazolin on two clinical strains Pseudomonas aeruginosa and Klebsiella pneumoniae producing NDM-1. Stability evaluation indicated that thioester 1 was partially hydrolysed by MβLs to be converted into the mercaptoacetic acid, revealing that the thioester and its hydrolysate mercaptoacetic acid jointly inhibit MβLs. Isothermal titration calorimetry (ITC) monitoring showed that thioester 1 exhibited dose-dependent inhibition on four MβLs tested, and the binding of 1/L1 showed mainly enthalpy driven, while 1/NDM-1 was found to be more entropy driven.Docking studies suggested that 1 bound to Zn(II) ion(s) preferentially via its carboxylate group, while other moieties interacted mostly with the conserved active site residues.

Key words antibiotic resistance; amino acid thioester; mercaptoacetic acid; broad-spectrum inhibitor; metallo-β-lactamase

Introduction

The β-Lactam antibiotics are the most important and most frequently used drugs for treating bacterial infections. However, the overuse of antibiotics has resulted in a large number of bacteria that are resistant to almost all antibiotics because of producing β-Lactamases. β-Lactamases are enzymes found in the periplasm which inactivate β-Lactam antibiotics by breaking the C–N bond of the β-Lactam ring, rendering the drugs ineffective (Chart 1A). More than 2000 distinct β-Lactamases are isolated. They are categorised into four classes: A–D, based on amino acid sequence similarity. Class B lactamases, known as metallo-β-lactamases (MβLs), utilise either one or two Zn(II) ions to catalyse the hydrolysis of β-Lactam antibiotics. MβLs are further divided into three subclasses B1–B3 on the basis of their sequence homologies, structures, and zinc ion contents, but the sequence similarity of the same subclass MβLs is less than 30%. Given the enormous biomedical importance of MβLs, a large amount of efforts have been made to develop MβL inhibitors. To date, at least three groups of MβL inhibitors have been reported: 1) Zinc binding inhibitors, such as N-heterocyclic dicarboxylic acid, azolylthioacetamides, triazolylthioacetamides, bisthiazolidines and maleic acid derivatives; 2) Zinc chelating inhibitors, represented as aspergillomarasmine A, [S,S]-ethylenediamine-N,N’-disuccinic acid (EDDS), and 1,4,7-triazacyclononane-1,4,7-triacetic

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Chart 1. (A) The Hydrolysis of Cefazolin by MβLs; (B) The Chemical Structures of β-Lactam Antibiotics and Amino Acid Thioesters

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acid (NOTA),\textsuperscript{13}) which inactivate clinically-relevant MβLs like VIM-2 and NDM-1 by sequestering both Zn(II) ions of each enzyme; and 3) covalent inhibitors such as ebselen, which inactivate NDM-1 by formation of an S–S bond with Cys221 residue at active site of the enzyme.\textsuperscript{14)} The compounds containing both thiol and carboxylic group are potent broad-spectrum inhibitors, such as L- and D-captopril,\textsuperscript{15–17)} thiomandelic acid,\textsuperscript{18)} mercapto-carboxylates,\textsuperscript{19)} and mercaptoacetic acid thioesters.\textsuperscript{20)} Among them, the thioesters were reported as “pro-drug” to deliver mercaptoacetic acid as the enzyme inhibitor.\textsuperscript{18,20)} Recently, our studies indicated that mercaptoacetate thioester is a highly promising scaffold for the development of L1 inhibitor with IC\textsubscript{50} values in submicromolar grade.\textsuperscript{21,22)}

However, almost all previous studies reported that the thioesters specifically inhibited the B3 subclass MβL L1, it is not clear whether the thioesters have inhibitory efficacy against the other subclasses MβLs. To probe the truth, in this work, nine amino acid thioesters were synthesized as we previously reported method\textsuperscript{22)} (Fig. 1), and tested as inhibitors against the purified MβLs IMP-1, VIM-2, Bla-2, NDM-1 and ImiS, which are the representative of the B1a, B1b and B2 subclass of MβLs, respectively. Also, antimicrobial activities of these inhibitors in combination with existing antibiotics against MβL-producing bacteria were evaluated. Isothermal titration calorimetry (ITC) was employed to characterize the binding of the inhibitor to MβLs. The stability of the thioesters in the presence of MβLs was investigated, and their action mechanism was studied by molecular docking.

Experimental

Materials Cefazolin and imipenem were purchased from Sigma (U.S.A.). All other chemicals used were of analytical grade. \textit{Escherichia coli} BL21 (DE3) cells were used for protein expression. The plasmids pET26b, pET24a and pET24b (Novagen) carrying the \textit{NDM-1}, \textit{IMP-1}, \textit{VIM-2}, \textit{Bla-2} or \textit{ImiS} gene were used for overexpression of the corresponding MβL. Amino acid thioesters 1–9 were synthesized as we described previously.\textsuperscript{22)}

Expression and Purification of MβLs \textit{E. coli} carrying \textit{NDM-1} plasmid was grown in LB medium with kanamycin (25 mg/L) at 37°C until they reached an OD\textsubscript{600} of 0.6, then 1 mM isopropyl β-D-thiogalactoside (IPTG) was added. Protein induction was performed for another 18–20 h at 25°C. Cells were harvested by centrifugation (5000 × \textit{g}, 20 min), resuspended in 50 mL of 30 mM Tris, pH 8.0 and disrupted with sonication for 40 min. The supernatant was collected by centrifugation (18000 × \textit{g}, 30 min) and dialyzed against the same buffer overnight. The dialysed supernatant was purified by a HiTrap Q sepharose XL fast protein liquid chromatography (FPLC) column, and the target protein was eluted at around 200 mM NaCl.

IMP-1, VIM-2, Bla2 and ImiS were purified in a similar manner as NDM-1. The target protein was purified by a HiTrap SP column in an FPLC system equilibrated with 30 mM Tris, pH 7.0 (ImiS, Bla2) or pH 7.5 (IMP-1, VIM-2). The concentration of proteins was determined by UV spectrophotometer according to Lambert–Beer’s Law and extinction coefficient of 27960 M\textsuperscript{−1} cm\textsuperscript{−1}/NDM-1, 49000 M\textsuperscript{−1} cm\textsuperscript{−1}/IMP-1, 54614 M\textsuperscript{−1} cm\textsuperscript{−1}/L1, and 37250 M\textsuperscript{−1} cm\textsuperscript{−1}/ImiS at 280 nm.

Enzyme Inhibition Assays The assays of enzyme activities were conducted in 30 mM Tris, pH 7.0. The concentrations of enzyme and substrate were 30 nM and 50 μM, respectively. The inhibitor concentrations causing 50% decrease in enzyme activity (IC\textsubscript{50}) were determined using kinetic assays by monitoring absorbance at 265 nm (for cefazolin) or 300 nm (for imipenem) on Agilent UV8453 spectrometer. The concentration of inhibitors was 50 μM for inhibition percentage screening, and ranging from 0.001 to 100 μM for IC\textsubscript{50} measurement. The IC\textsubscript{50} values were derived by the non-linear least-squares fitting of the data employing an algorithm implemented in the GraphPad Prism 5.0.

Stability Analysis The stability of amino acid thioester 1 in the presence and absence of MβL was evaluated by HPLC performed on an LC-20A HPLC (Shimadzu, Kyoto, Japan). The HPLC used a Kromasil C18 column (4.6 × 250 mm i.d.) with 0.1% trifluoroacetic acid (TFA) in H\textsubscript{2}O as mobile phase A and 40% acetonitrile and 0.1% TFA as mobile phase B. The column was eluted in a linear gradient at a flow rate of 1 mL/min for 20 min. The UV-Vis detection wavelength was set at 254 nm and the injection volume was 10 μL. The retention time of the thioester 1 and its hydrolysate was 16.7 and 17.7 min, respectively.

Ellman’s reagent was also used to monitor the stability of thioester by detecting the formation of 2-nitro-5-thiobenzoic acid (TNB) at 412 nm with a molar extinction coefficient of 14150 M\textsuperscript{−1} cm\textsuperscript{−1} (\textsuperscript{23,24)} (Fig. S5). The inhibitor 1 was premixed with L1 for 0–12 h. A 50 μL of 4 mg/mL Ellman’s reagent, 2.5 mL of 0.1 M sodium phosphate (pH 8.0) and 250 μL of pre-incubated inhibitor and L1 were mixed, incubated at room temperature for 15 min, and then the absorbance was measured at 412 nm. The concentration of formed TNB was determined using Lambert–Beer’s Law, which is equal to the concentration of the free sulfhydryl in the sample.

ITC Characterization For ITC characterization, the
MβLs samples, substrates, and inhibitor were prepared with 30mM Tris, pH 7.0, with 1% addition of dimethyl sulfoxide (DMSO). In the single injection mode, the concentration of MβLs used was 10nM, the substrate was 1mM, and inhibitor was varied from 0.5 to 500µM. Prior to the injection, the enzyme, inhibitor, and substrate were degassed with centrifugation in a speed of 14000 × g for 5 min. The enzyme sample was pre-incubated with different concentrations of inhibitor for 30 min and then delivered into the sample cell (210µL). A 38µL of 1mM substrate (cefazolin for NDM-1, IMP-1 and L1, and imipenem for ImiS) in the syringe was titrated into the sample cell in a single injection mode. The reaction in the absence of inhibitor was measured as the control. Heat flow (µcal/s) was recorded as a function of time. Data were collected every 1 s until the signal reached the baseline and continued to be recorded for an additional 60–100 s to generate the final baseline. The collected data was analysed with MicroCal Analysis Launcher Origin 7.0 software.

For the characterization of inhibitor binding to MβLs by ITC, the multiple injection mode was performed. The tested compound and protein were dissolved in 30mM Tris, pH 7.5, with 1% addition of DMSO, respectively. The compound was titrated into a solution of protein, over 26 aliquots of 1.5µL (the first injection was 0.5µL) with 150 s between injections. The concentrations of protein and ligand were 50µM and 0.5mM, respectively. For the characterization of Zn(II) ion(s) binding to inhibitors, the same multiple injection program was used as above. The concentration of zinc sulfate was 2mM, corresponding to 0.2mM inhibitors.

Docking Studies The docking studies were performed using Autodock 4.0, which employs the optimization algorithm in its scoring function. The ChemBio 3D programme was used to construct the structure of compounds, and all possible torsion angles in the compounds were set free to carry out flexible docking. The inhibitor was docked into the active site of the crystal structures of NDM-1 (PDB code 4EYF), IMP-1 (PDB code 5EWA), CphA (PDB code 2QDS), and L1 (PDB code 2AIO). There is no high-resolution crystal structure of ImiS available, so a very closely related structure (96% sequence identity) of CphA was used instead. The binding energies, hydrophobic contacts and hydrogen bonds between each protein and inhibitor can be obtained from the docking studies. The protein structures were visualized and analysed with PyMOL (Delano Scientific).

**Cytotoxicity Evaluation** The methyl thiazolyltetrazolium (MTT) assay was used to analyse cell cytotoxicity. The L929 mouse fibroblast cell line was cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) medium (10% fetal bovine serum (FBS)) in 5% CO2 at 37°C for 24h. The cells were then placed in a 96 well plate at a cell density of 6 × 10^4 cell/mL with a 100µL/well. After incubation at 37°C in 5% CO2 for 24h, the medium was replaced with DMEM medium plus 10% FBS.

**Table 1. Inhibitory Activity (IC_{50}, µM) of Amino Acid Thioesters against MβLs**

| Compound | NDM-1 | IMP-1 | ImiS | L1 |
|----------|-------|-------|------|----|
| 1        | 16.6 ± 0.5 | 12.0 ± 0.2 | 0.02 ± 0.01 | 0.08 ± 0.01 |
| 2        | 27.9 ± 1.2 | 29.6 ± 1.2 | 0.45 ± 0.02 | 0.21 ± 0.03 |
| 3        | 54.9 ± 1.0 | 29.3 ± 0.8 | 1.24 ± 0.10 | 0.60 ± 0.09 |
| 4        | 44.0 ± 0.8 | 25.7 ± 0.9 | 0.92 ± 0.08 | 0.14 ± 0.01 |
| 5        | >200     | 28.4 ± 1.0 | 0.46 ± 0.03 | 0.24 ± 0.03 |
| 6        | 28.6 ± 0.2 | 36.5 ± 0.9 | 1.00 ± 0.05 | 0.32 ± 0.02 |
| 7        | >200     | 44.6 ± 5.0 | 1.62 ± 0.40 | 0.02 ± 0.01 |
| 8        | 19.7 ± 0.5 | 20.4 ± 0.5 | 2.62 ± 0.20 | 0.09 ± 0.01 |
| 9        | 37.7 ± 0.4 | 32.4 ± 0.7 | 0.02 ± 0.01 | 0.07 ± 0.01 |

The substrate for NDM-1, IMP-1 and L1 was cefazolin, for ImiS was imipenem; Results represent means ± standard deviations of triplicate experiments.

Fig. 2. Michaelis–Menten Plots of NDM-1 (A)/IMP-1 (B)/ImiS (C)/L1 (D) Catalysed Hydrolysis of Substrate (Cefazolin or Imipenem) in the Presence of Inhibitor 1 at Various Concentrations

Concentrations of inhibitor 1 were 0/125/250/500µM (NDM-1), 0/100/250/500µM (IMP-1), 0/0.02/0.04/0.08µM (ImiS), and 0/0.2/0.5/1.0µM (L1).
containing different concentrations of inhibitor 1, 5 and 9 (0, 6.25, 12.5, 25, 50, 100 and 200 μM). After incubation for 24h, 20 μL of the MTT solution was added to each well and the plate was incubated in 5% CO₂ at 37°C for 4h. Finally, the supernatant was discarded and 100 μL of DMSO was added to the wells to measure absorbance of solubilizing the formazan at 570 nm. All experiments were conducted in triplicate.

**Minimal Inhibitory Concentration (MIC) Determination**

The MIC of antibiotic against bacteria in the presence of enzyme inhibitor was determined according to the microdilution method of the CLSI.²⁶ Four *E. coli* BL21 cells expressing NDM-1, IMP-1, ImiS or L1 were used to assess the inhibitor. Three *E. coli* DH10B cells harbouring the recombinant pBCKS-NDM-1/ImiS/L1 plasmid and two clinical stains expressing NDM-1 were also used. The MIC values were determined at a concentration of 128 μg/mL inhibitor in combination with cefazolin or imipenem, respectively. The MIC assays were performed in triplicate.

**Results**

**Enzyme Inhibition Studies** To investigate whether these amino acid thioesters were broad-spectrum inhibitors of MβLs, the inhibitory activity of the thioesters against NDM-1 (B1b), IMP-1 (B1a), VIM-2 (B1a), Bla2 (B1a), and ImiS (B2) was measured as described previously.²⁷ Percent inhibition gained (Figure S1) showed that all amino acid thioesters tested exhibited more than 50% inhibition on NDM-1, IMP-1 and ImiS (except 5 and 7 on NDM-1) at an inhibitor concentration of 50 μM. The IC₅₀ values of 1–9 against NDM-1, IMP-1 and ImiS were further determined, and the data (Table 1) revealed that all of these thioesters exhibited inhibitory activity on ImiS, IMP-1, and NDM-1, with an IC₅₀ range of 0.02–2.62, 12.0–44.5, and 16.63–54.9 μM (except 5 and 7 on NDM-1), and 1 was found to be the best inhibitor with an IC₅₀ value of 20 nM on ImiS. Given the best and broad-spectrum inhibition of 1 on MβLs, 1 was chosen for further functional and mechanism studies.

The inhibition modes and Kᵢ values of 1 against NDM-1, IMP-1, ImiS and L1 were determined by fitting to the Michaelis–Menten equation through nonlinear fitting with SigmaPlot 12.0 (Fig. 2). The results showed that 1 exhibited competitive inhibition against NDM-1, IMP-1 and L1, but an uncompetitive inhibition on ImiS. The Kᵢ values were determined to be 0.06 ± 0.02, 0.18 ± 0.07, 20 ± 4, and 88 ± 6 μM for ImiS, L1, IMP-1, and NDM-1, respectively (average ± standard deviation (S.D.) of triplicates).

**Stability Assay** In order to clarify the action mechanism of amino acid thioesters against MβLs that the thioester own, or its hydrolysat mercaptoacetic acid inhibited the MβLs, HPLC was employed to monitor the hydrolysis of inhibitor 1 in the presence of NDM-1, IMP-1, ImiS and L1, respectively. The monitoring results showed that about 20% of 1 was hydrolysed by MβLs after incubation of it with the enzymes for 1h (within the time of IC₅₀ measurement) (Table 2, Fig. S2), but the hydrolysis of 1 was not detected in the absence of MβL.

To further confirm the results from HPLC above, Ellman’s reagent was used to quantitate the free sulfhydryl in the hydrolysate product after incubating with L1. The concentration-time dependence of the mercaptoacetic acid from 1 hydrolysis by MβLs was showed in Fig. 3. It is clearly observed that the concentration of mercaptoacetic acid was 23.0 μM after inhibitor 1 was incubated with L1 for 1h, meaning that 23% of the thioester was hydrolysed, which is consistent with the HPLC results.

**ITC Characterization** ITC is a potent approach to measure the heat energy associated with a molecular interaction and was to assess the activity of enzyme inhibitors. The ITC with a single injection mode was employed to evaluate the inhibition of amino acid thioester on MβLs. The heat flows (μcal/s) of antibiotics hydrolysis catalysed by MβLs in the presence of inhibitor 1 at different concentration are shown in Fig. 4. It shows the negative thermal power (P) after the antibiotics injection, indicating that the hydrolysis reaction is exothermic and the hydrolysis of cefazolin and imipenem is inhibited by adding 1 into the reaction mixtures.

To evaluate the binding of Zn(II) ions by thioester 1 and its hydrolysate mercaptoacetic acid, calorimetric experiments were performed by ITC using a multiple injection mode. A solution of zinc sulfate was titrated into the sample cell containing 1 or mercaptoacetic acid, and the binding heat was monitored. As shown in Fig. S3, 1 exhibited low binding affinity for Zn(II) ions with a K₅ value of 347 ± 9.9 μM, which is much larger than the data of mercaptoacetic acid chelating Zn(II) ions (K₅ = 45 ± 4.9 μM).²⁸

To investigate the thermodynamic behaviour of the interaction of MβL with inhibitor 1, the ITC with multiple injection mode was performed by directly titration of ligand solution into protein (Fig. 5). The thermodynamic parameters, including enthalpy change (ΔH) and entropy change (ΔS) (listed in Table S1), were determined, while the Gibbs free energy (ΔG) was calculated with formula ΔG = ΔH − TΔS. The Kₛ values of I/L1 and I/NDM-1 binding are 9.6 and 61.7 μM, respectively, which is consistent with the IC₅₀ data (0.08 and 16.6 μM) of I against L1 and NDM-1, suggesting that the binding of I/L1 is mainly enthalpy driven, but the binding of I/NDM-1 is more entropy driven.

**Docking Studies** To explore potential binding modes
of amino acid thioesters alone as potent MβLs inhibitor, we
performed molecular docking of inhibitor 1 into the active
sites of NDM-1, IMP-1, CphA, and L1 using the same pro-
cedure as reported previously.7) The conformations shown in
Fig. 6 are the lowest-energy conformations of those clusters,
with the binding energy of −11.63, −11.41, −12.88, and
−12.18 kcal/mol for the NDM-1/1, IMP-1/1, CphA/1, and L1/1
complexes, respectively.

As shown in Figs. 6B, 6C and 6E, one of the carboxyl
oxygen (O1) of 1 bridges two Zn(II) ions, while another
oxygen(O2) coordinates Zn(II) ion and forms hydrogen bonds
with Lys224 (NDM-1, IMP-1) or Ser221 (L1) (BBL standard
number used throughout29). In the NDM-1/I and IMP-1/I
complexes, the hydrophobic interactions between the R1 group
of 1 (Chart 1B) and residues from the L3 loop (Met67NDM-1,
Val73NDM-1, Trp64IMP-1 and Val67IMP-1) have been observed.
The L3 loop, which is a feature of most B1 MβLs, has been
hypothesized to contribute to substrate binding specifically.30)
It can be observed in Fig. 6E that the L1/I complex resembles
the binding mode of mercaptoacetic acid thioesters reported
previously,21,22) while the R2 group nitrogen (N2) of 1 forms
double hydrogen bonds with Tyr32, probably due to big 4-biphenyl.
In addition, the sulfur atom (S1) forms hydrogen bonds with
Ser223, and the side chain of 1 is bound to the hydrophobic

Fig. 4. Overlaid Heat Flow Curves of Cefazolin or Imipenem Hydrolysis by NDM-1 (A), IMP-1 (B), ImiS (C), and L1 (D) in the Presence of 1 at a
Concentration Ranging from 0 to 500 µM Using a Single Injection Mode at 25°C Monitored by ITC
The concentration of antibiotic was 1 mM, and the concentration of NDM-1, IMP-1, ImiS and L1 was 10 nM.

Fig. 5. ITC Profiles of Titration of a Solution of Inhibitor 1 into an NDM-1 (A) or L1 (B) Protein Solution at 25°C
The concentrations of 1 and protein were 0.5 mM and 50 µM, respectively. The insets in A and B show the values of ΔG, ΔH and −TΔS.
pocket formed by Val34, Trp38, Pro226, and Lys300, which stabilizes the complex, revealing why 1 exhibits a low IC$_{50}$ against L1.

In the CphA/1 complex shown in Fig. 6D, the carboxyl oxygen (O1) of 1 coordinates Zn(II) ion at Zn2 site with a distance of 1.3 Å, and another oxygen (O2) forms a hydrogen bond with Lys224 with a distance of 2.6 Å. This binding mode is reminiscent of the interaction between CphA and hydrolysed biapenem reported earlier. 31) Also, the nitrogen (N2) on R$_2$ interacts with Thr157 and oxygen (O3) with Asn233. The former interaction is usually made by C7 carboxyl group of β-Lactam,31) while the latter is reminiscent of the interaction between the β-Lactam carbonyl and Asn233, which has been proposed to be part of an oxyanion hole. 7,32) A hydrophobic interaction between R$_2$ and Val67 and Trp87 is also observed.

**Toxicity Evaluation** The potential toxicity of inhibitors is a major concern for their biomedical applications. To determine the maximum non-toxic concentrations of the amino acid thioesters against mammalian cells, different concentrations of 1, 5 and 9 were incubated with L929 mouse fibroblast cell line and cell viability was evaluated using the MTT reagent. The results were expressed as percent viability of each sample compared with that of untreated control cells (PBS buffer, pH 7.4). As shown in Figure S4, the cell viability was over 80% in the presence of 1, 5 and 9 at a dose up to 200 µM, illustrating the amino acid thioesters had little influence on cell proliferation.

**MIC Determination** The ability of the MβLs inhibitors to restore the antimicrobial activity of antibiotics against bacteria expressing MβL was investigated by determining the MICs of the antibiotics in the absence and presence of 128 µg/mL thioesters. The data listed in Table 3 shows that inhibitors 1, 5 and 9 increased the antimicrobial effect of antibiotic(s) (cefazolin and/or imipenem) against both *E. coli* BL21 and *E. coli* DH10B strain expressing ImiS, L1, IMP-1, and NDM-1 (except 5), resulting 2- to 32-fold reduction in MIC. Also, 1 restored 2- and 4-fold antimicrobial activity of cefazolin against clinical strains *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* producing NDM-1, respectively, but 5 and 9 did not.

Table 3. MICs of Cefazolin and Imipenem against *E. coli* Cells Expressing MβLs and Their Parent Strain in the Presence and Absence of Amino Acid Thioesters 1, 5 and 9

| *E. coli* strains | Antibiotic (µg/mL) | +1 | +5 | +9 |
|------------------|------------------|----|----|----|
|                  |                  |    |    |    |
| *E. coli* BL21 (NDM-1)\(^a\) | 64 | 32 | 64 | 32 |
| *E. coli* BL21 (IMP-1)\(^a\) | 128 | 32 | 64 | 64 |
| *E. coli* BL21 (ImiS)\(^b\) | 64 | 2 | 8 | 4 |
| *E. coli* BL21 (L1)\(^a\) | 128 | 8 | 16 | 8 |
| *E. coli* DH10B (NDM-1)\(^a\) | 32 | 8 | 32 | 16 |
| *E. coli* DH10B (ImiS)\(^b\) | 16 | 4 | 8 | 16 |
| *E. coli* DH10B (L1)\(^a\) | 64 | 16 | 32 | 32 |
| *P. aeruginosa* (NDM-1)\(^a\) | 32 | 16 | 32 | 32 |
| *K. pneumoniae* (NDM-1)\(^a\) | 32 | 8 | 32 | 32 |

\(^a\) The antibiotics used were cefazolin and \(^b\) imipenem, respectively. 1, 5 and 9 in combination with antibiotic were tested at a concentration of 128 µg/mL.
Discussion

The present study revealed that nine amino acid thioesters tested inhibited NDM-1, IMP-1, ImiS and L1 from three subclasses MβLs, indicating that the thioesters are the broad-spectrum inhibitors of MβLs. Comparison of the I_{50} values listed in Table 1, clearly, the thioesters exhibited better inhibitory efficacy on L1 than on ImiS, than on IMP-1, and than on NDM-1. The thioester 1 showed uncompetitive inhibition against ImiS, but competitive inhibition on NDM-1, IMP-1 and L1.

Payne et al. reported that the mercaptoacetic acid thioester irreversibly inhibit β-Lactamase BcII, due to a mercaptoacetic acid was delivered to the metal-coordinating cysteine as a result of the thioester conversion by the enzyme,20 but ITC studies indicated that inhibition was not accomplished through chelation of the Zn(II) ions.33 While Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) spectral characterization showed that the mercaptoacetate covalently bound to L1.34 These reports indicated that the essence of thioesters inhibiting L1 is the contribution of either the thioester itself or its hydrolysate mercaptoacetic acid. In this study, HPLC and Ellman analysis indicated that around 20% amino acid thioesters were hydrolysed after incubation of it with MβL for 1 h, suggesting that both thioester itself and its hydrolysate mercaptoacetic acid jointly inhibit MβLs, which is consistent with the results Chen et al. reported.35

The ITC characterization with a single injection mode showed that the hydrolysis of cefazolin and imipenem was inhibited by adding the thioester 1 to the reaction mixtures. The higher the inhibitor concentrations were, the lower the hydrolysis rates of antibiotics were (Fig. 5), confirming thermodynamically that the activity of NDM-1, IMP-1, ImiS and L1 was inhibited by the thioester 1, and the inhibition was dose-dependent. Titration of Zn(II) ions into a solution of inhibitor (multiple injections) suggested that the inhibition of thioester 1 on MβLs was not realized through chelating the Zn(II) ions of enzyme active sites (Fig. S3). The thermodynamic data gained by ITC characterization with multiple injection mode through injection of 1 into MβL indicated that the binding between 1/L1 shows mainly enthalpy driven and dominated by van der Waals interactions, along with hydrogen bonding, for the values of ΔH and ΔS were negative, while 1/NDM-1 was found to be more entropy driven and based on hydrophobic interaction, because ΔS is positive30 (Fig. 5).

The antibacterial activity of amino acid thioesters on bacteria expressing MβLs was investigated by determining the MICs of antibiotics in the absence and presence of 1, 5 and 9. It is notable that the thioester 1 exhibited the best activity against the antibiotic resistant strains harbouring B1, B2 and B3 subclass MβLs. The synergy of 1 with imipenem restored 32-fold antibacterial effect of the drug on E. coli BL21 strain expressing ImiS. Also, thioester 1 was able to restore the activity of cefazolin on both clinical isolates K. pneumoniae and P. aeruginosa expressing NDM-1. Also, the inhibitors 5 and 9 resulted in a 1–16-fold reduction of cefazolin and/or imipenem in MIC. These results are consistent with the inhibitory efficacy of the thioester against MβLs in enzyme inhibition assays.

The docking studies, in agreement with the results of zinc binding ability of inhibitor 1 determined by ITC, show that the inhibition effect is achieved by coordination with Zn(II) ion(s), formation of the hydrogen bond, and hydrophobic interaction, instead of cheating Zn(II) ion(s). Thioester 1 binds Zn(II) ion(s) preferentially via its carboxyl, while other moieties interact mostly with the conserved active site residues Lys224 (NDM-1, IMP-1 and ImiS) or Ser221 (L1). 36 These reports indicated that around 20% amino acid thioesters were hydrolysed after incubation of it with MβL for 1 h, suggesting that both thioester itself and its hydrolysate mercaptoacetic acid jointly inhibit MβLs, which is consistent with the results Chen et al. reported.35

Conclusion

Based on the structural similarity to the β-Lactam, nine amino acid thioesters 1–9 were developed and evaluated with MβLs from three subclasses enzymes. These molecules exhibited broad-spectrum inhibitory efficacy against ImiS (B2), IMP-1 (B1a), NDM-1 (B1b), and L1 (B3), with I_{50} values range of 0.02–2.62, 12.0–44.5, 16.63–54.9, and 0.02–0.6 µM (except 5 and 7 on NDM-1), respectively, and 1 was found to be the best broad-spectrum inhibitor with an IC_{50} of 20 nM on ImiS. The inhibitors 1, 5 and 9 restored 2–32-fold antibacterial activity of antibiotic against recombinant E. coli expressing ImiS, L1, IMP-1 and NDM-1 (except 5 on NDM-1), and also, the most promising inhibitor 1 showed antimicrobial activity against two clinical strains P. aeruginosa and K. pneumoniae producing NDM-1. Stability evaluation indicated that thioester 1 was partially hydrolysed by MβLs to be converted into the mercaptoacetic acid, revealing that the thioester and its hydrolysate mercaptoacetic acid jointly inhibit MβLs. ITC monitoring showed that 1 exhibited dose-dependent inhibition on four MβLs tested, and the thioester and its hydrolysate mercaptoacetic acid inhibit MβLs in a different manner. Docking studies revealed that 1 binds zinc(II) ion(s) preferentially via its carboxyl, while other moieties interact mostly with the conserved active site residues Lys224 (NDM-1, IMP-1 and ImiS) or Ser221 (L1). The information gained in this work reveals that the amino acid thioesters have the potential to develop to be broad-spectrum inhibitors of MβLs.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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