Inhibition Studies on the Metallo-β-lactamase L1
from Stenotrophomonas maltophilia

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Received January 29, 1999 and in revised form May 5, 1999

In an effort to identify a competitive inhibitor that can be used in future spectroscopic and crystallographic studies and to better understand the interaction of a mercaptoacetic acid-thiolester-containing compound with metallo-β-lactamase L1 from Stenotrophomonas maltophilia, inhibition studies using two thiol-containing compounds were conducted. N-(2'-Mercaptoethyl)-2-phenylacetamide is a competitive inhibitor of L1 with a $K_i$ of $50 \pm 3 \mu M$, and this compound is not a time-dependent inactivator of L1. N-Benzylacetyl-D-alanylthioacetic acid is a competitive inhibitor of L1 with a $K_i$ of $1.6 \pm 0.3 \mu M$. Matrix-assisted laser desorption ionization time-of-flight mass spectrometric studies revealed that 2 mol of mercaptoacetate covalently bind to L1 upon incubation of the enzyme with N-benzylacetyl-D-alanylthioacetic acid; however, this covalently modified enzyme has the same activity as wild-type L1. Last, inhibition studies were used to demonstrate that 4-morpholinoethanesulfonic acid does not inhibit L1, even at concentrations up to 300 mM. This work identifies two possible competitive inhibitors which can be used in future structural studies and further demonstrates inhibitory heterogeneity among the metallo-β-lactamases.

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Key Words: metallo-β-lactamase; L1; Stenotrophomonas maltophilia; inhibition.

The emergence of pathogenic bacteria that are resistant to antibiotics is a very important biomedical problem facing society. These bacteria have become resistant by acquiring the ability to bind to and inactivate the antibiotic or to transport the antibiotic out of the cell (1). One of the most common strategies for resistance to β-lactams is for the bacterium to produce a β-lactamase, which catalyzes the hydrolysis of penicillins and cephalosporins. Most of these β-lactamases utilize an active-site serine for nucleophilic attack on the β-lactam carbonyl (2, 3); however, recently, a new group of β-lactamases that require Zn(II) for activity and do not utilize a serine group for nucleophilic attack have emerged. These newer enzymes, metallo-β-lactamases, require 1–2 Zn(II) ions per protein monomer, are not inhibited by any known clinically useful compounds, and are found in a number of organisms, several of which are minor pathogens, including Serratia marcescens, Stenotrophomonas maltophilia, Pseudomonas aeruginosa, and Bacteroides fragilis (4, 5).

Previous kinetic, biochemical, inhibition, and structural studies suggest significant differences between the enzymes from the various sources. For example, the Aeromonas hydrophila enzyme requires only one Zn(II) for full catalytic activity (6), while the other characterized metallo-β-lactamases require two Zn(II) per monomer (7–10). The steady-state kinetic constants for the enzymes vary by several orders of magnitude (11–13); in fact, the substrate specificities of the enzymes differ, with the enzymes from Aeromonas species significantly hydrolyzing only carbapenems while the other enzymes hydrolyze penicillins and cephalosporins best (13, 14). Recent crystal structures of the metallo-β-lactamases from B. fragilis, Bacillus cereus, and S. maltophilia reveal very significant structural differences between the enzymes and that the S. maltophilia enzyme utilizes a different metal binding motif than the other enzymes (9, 15–18). The metallo-β-lactamases from the different sources demonstrate differing efficacies toward nonclinically useful inhibitors such as a group of mercaptoacetic acid-containing species and biphenyl tetrazoles (18, 19). These structural and mechanistic differences between the metallo-β-lactamases suggest that one inhibitor may not inhibit all metallo-β-lactamases. The prevalence of these en-
zymes in pathogens and the certain emergence of the metallo-β-lactamases in other bacteria necessitate a survey study of several of the enzymes, particularly those enzymes that appear to be from different classes. It is uncertain which bacterium will provide a major pathogen with the genetic information necessary to produce a metallo-β-lactamase.

This work describes inhibition studies on the metallo-β-lactamase L1 from S. maltophilia.

**EXPERIMENTAL PROCEDURES**

General. The metallo-β-lactamase L1 from S. maltophilia was overexpressed and purified as previously described (7). All kinetic studies were conducted on a Hewlett-Packard 5480A UV–Vis diode-array spectrophotometer at 25°C. The buffer used in all kinetic studies was 50 mM cacodylate, pH 7.0, and the substrate was nitrocefin. Working stocks of nitrocefin were prepared by dissolving nitrocefin (Becton–Dickinson) in 10 mM of 50 mM cacodylate, pH 7.0, and filtering the resulting solution through a 0.45-µm syringe filter to remove any undissolved particulates. 3H NMR spectra were collected on a Bruker spectrometer operating at 200 MHz. The structures of the inhibitors used in this study are shown in Fig. 1.

Synthesis of N-(2'-mercaptoethyl)-2-phenylacetamide (MEPA)² (20). MEPA was synthesized as described previously (20), except that after separation of the product by TLC, it was recrystallized from hot ethanol. A concentrated stock of N-(2'-mercaptoethyl)-2-phenylacetamide was made by dissolving 5–10 mg of the compound in 1 mL of ethanol, and a working stock of the inhibitor was made by diluting the concentrated stock with 50 mM cacodylate, pH 7.0.

Synthesis of N-benzylacetyl-D-alanylthioacetic acid (BATA) (19). N-Benzyl-D-alanine (3.88 mmol) was added to 35 mL dimethylformamide-N-acetylacetate (1:1) containing ethyl chloroformate (3.88 mmol) and triethylamine (7.76 mmol) with stirring at 25°C. After 15 min, the solution changed into a yellow color, and mercaptoacetic acid (3.88 mmol) and triethylamine (3.38 mmol) were added. The reaction mixture was acidified with 2 N HCl (2 mL), and the volatile components were removed by rotary evaporation. The resulting white residue was stirred with 30 mL of acetone, and a white precipitate formed and was filtered. The filtrate was vaporized to give the crude product, which was separated by silica gel chromatography (50% methanol/water) and further purified by reversed-phase partition chromatography with HPLC–RP18F plates in 0.1% trifluoroacetic acid–acetonitrile. N-Benzylacetyl-D-alanylthioacetic acid was isolated as a yellow oil in an overall yield of 82%. 1H NMR (CDCl3) δ 1.59 (3H, q, CH3), 3.76 (2H, t, CH2), 4.84 (1H, d, CH), 7.45 (5H, m, Ar-H), 7.80 (1H, t, NH), 9.88 (1H, s, COOH).

Mass spectrometry. Mass spectra were acquired on a Bruker (Billerica, MA) Reflex II time-of-flight mass spectrometer operating in the linear mode. Ions were produced by matrix-assisted laser desorption ionization (MALDI) employing the 355-nm line of a New Wave Research (Sunnyvale, CA) Minilase-10 Nd: YAG laser. The matrix solution for MALDI was saturated α-cyano-4-hydroxycinnamic acid in a solvent system of acetonitrile/water (70/30, v/v) with 0.01% trifluoroacetic acid. The enzyme–BATA reaction mixture was mixed with the matrix solution at a ratio of about 2:5. Bovine serum albumin (BSA) and cytochrome c were used as external mass calibrants. The [M +2H]⁺ and [M +3H]⁺ of BSA (m/z 32,216 and 22,144, respectively) bracketed the enzyme’s measured m/z of 28,670.

Inactivation studies. A sample of 317 µM L1 was diluted 100-fold using different volumes of 1.08 mM N-(2'-mercaptopethyl)-2-phenylacetamide or 380 µM N-benzylacetyl-D-alanylthioacetic acid and 50 mM cacodylate, pH 7.0. The reaction of the thiol-containing compounds with L1 was allowed to proceed for 30 min on ice. A parallel set of reactions was prepared using the same thiol-containing compounds and 50 mM cacodylate, pH 7.0, containing 100 µM ZnCl2. Ten-microliter aliquots were taken from the reaction mixtures and pipetted into a cuvette containing 940 µL of cacodylate buffer and 50 µM of 713 µM nitrocefin. The formation of hydrolyzed nitrocefin was followed at 485 nm, and the data were used to calculate rates as described previously (7).

Inhibition studies. N-(2'-Mercaptopethyl)-2-phenylacetamide and N-benzylacetyl-D-alanylthioacetic acid inhibition studies were conducted in 50 mM cacodylate buffer, pH 7.0, at 25°C with nitrocefin as the substrate and working stocks of 5.25 mM MEPA and 380 µM BATA as the inhibitors. Nitrocefin concentrations were varied between 0 and 525 µM and 0 and 10 µM for MEPA and BATA, respectively. The mode of inhibition was determined by generating Lineweaver–Burk plots of the data, and the K_i values for the inhibitors were determined by fitting initial velocity versus substrate concentration at each inhibitor concentration to

\[ \frac{V_i}{V} = \frac{K_{mi}}{[S]} + \frac{K_{mi}}{[I]K_i} \]

where \( V_i \) is initial velocity, \( V_{max} \) is maximum velocity, [S] is initial substrate concentration, \( K_{mi} \) is Michaelis constant, [I] is inhibitor concentration, and K_i is inhibition constant. The reported K_i values represent the averages of all calculations with each inhibitor, and the reported errors are standard deviations (\( \sigma_{K_i}, n = 9 \)). Each set of inhibition studies was conducted in triplicate.

Inhibition studies using 4-morpholinoethanesulfonic acid (MES) as a potential inhibitor were conducted in 50 mM cacodylate buffer, pH

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² Abbreviations used: MEPA, N-(2’-mercaptopethyl)-2-phenylacetamide; MALDI, matrix-assisted laser desorption ionization; BATA, N-benzylacetyl-D-alanylthioacetic acid; BSA, bovine serum albumin; TOF, time-of-flight.
RESULTS

Studies Using N-(2'-Mercaptoethyl)-2-Phenylacetamide as an Inhibitor

Recently, Page and co-workers reported that N-(2'-mercaptoethyl)-2-phenylacetamide is a competitive inhibitor of β-lactamase L1 from B. cereus with a K_i of 70 μM (20). In order to determine whether this compound is also an inhibitor of L1, we synthesized MEPA as described by Page and co-workers (20). Steady-state kinetic studies using concentrations of N-(2'-mercaptoethyl)-2-phenylacetamide ranging from 0 to 270 μM inhibitor demonstrated that MEPA is a competitive inhibitor of L1 with a K_i of 50 ± 3 μM (data not shown).

To probe whether MEPA could inactivate L1 over time, we incubated L1 with concentrations of MEPA varied from 0 to 350 μM for 30 min and assayed the enzyme after the incubation. Under these conditions, there was no loss in enzyme activity after incubation with MEPA in the presence or absence of 100 μM Zn(II).

Studies Using N-Benzylacetyl-D-alanylthioacetic Acid as an Inhibitor

Previously, Payne et al. reported that N-benzylacetyl-D-alanylthioacetic acid (SB214752) inhibited L1 with an IC_{50} of 2–3 μM (19). To further characterize the interaction of this inhibitor with L1, steady-state inhibition studies were conducted. These studies using 7 to 72 μM nitrocefin as substrate and 0 to 7.6 μM N-benzylacetyl-D-alanylthioacetic acid as the inhibitor demonstrated that BATA is a competitive inhibitor of L1 with a K_i of 1.6 ± 0.3 μM (Fig. 2).

Studies were also conducted to determine whether BATA inactivates L1 over time. After a 30-min incubation of L1 with concentrations of BATA ranging from 0 to 4.0 μM, activity assays of the resulting reaction mixture demonstrated that no time-dependent inactivation of L1 occurred in the absence or presence of 100 μM Zn(II).

Payne et al. previously reported that several mercaptoacetic acid thioesters irreversibly inhibited β-lactamase L1 from B. cereus by a mechanism-based delivery of mercaptoacetic acid to the metal binding cysteine ligand in the enzyme (19). To probe whether a similar covalent modification of a cysteine residue in L1 occurs, equimolar samples of 38 mM N-benzylacetyl-D-alanylthioacetic acid and 68 μM L1 were mixed and allowed to react for 30 min at 22°C. MALDI–TOF mass spectrometry was used to detect the formation of any covalently attached mercaptoacetates to L1. The mass spectrum of isolated L1 with the [M+H^+] peak at 28,670 m/z is shown in Fig. 3A and is very similar to that previously published (7). The MALDI–TOF mass spectrum of the BATA–L1 reaction mixture is shown in the inset to Fig. 3B. The largest peak is attributable to L1, while the shoulder at 28,850 m/z is attributable to L1 with two attached mercaptoacetates (+180 m/z). To probe the nature of this attachment, the L1–BATA was dialyzed against 3 × 1 L of 50 mM HEPES, pH 7.5, at 4°C for 2 days. Activity assays of the resulting solution demonstrated that the dialyzed L1–BATA sample had the same activity as a control L1 sample that was dialyzed but not reacted with BATA. A MALDI–TOF spectrum of the dialyzed L1–BATA sample was identical to that of L1–BATA (Fig. 3B).

Inhibition Studies with L1 and Mes

Recently, Fitzgerald et al. reported that Mes is a weak, competitive inhibitor of the metallo-β-lactamase CcrA from B. fragilis with a K_i of 23 mM (17). To determine whether Mes also inhibited L1, inhibition studies were conducted using concentrations of Mes varying between 0 and 300 mM. There was no inhibition of L1 observed using these concentrations of Mes.

DISCUSSION

The discovery of clinically useful inhibitors is one of the most important reasons for studying metallo-β-lactamases. The administration of such an inhibitor along with a currently available penicillin, cephalosporin, or carbapenem to a patient with a β-lactam-resistant bacterial infection could prove to be an effective therapy. A similar approach has been used to treat bacterial infections in which the bacteria produce serine-β-lactamases. An example of an inhibitor of the
FIG. 3. MALDI–TOF mass spectrum of (A) 3.4 μM L1 and (B) 3.4 μM L1 and 1.4 mM N-benzylacetyl-o-alanythioacetic acid.
Class A β-lactamases is clavulanic acid, which has been administered with amoxycillin (Augmentin) or ticarcillin (Timentin) to combat penicillin-resistant bacterial infections (21). To date, all of the serine β-lactamase inhibitors have been shown to be ineffective at inhibiting metallo-β-lactamases; in fact, clavulanic acid is rapidly hydrolyzed by all tested metallo-β-lactamases (5, 7, 22, 23). Nonetheless, several nonclinically useful inhibitors have been reported, and structural and mechanistic information from the study of these inhibitors may aid in the design of clinically useful compounds. In addition, the discovery of competitive inhibitors of the metallo-β-lactamases will aid in the better understanding of enzyme structure/function because such an inhibitor could be used in crystallographic or spectroscopic studies.

One of the main goals of our research is to understand the structure of L1 during catalysis and substrate binding. A commonly used strategy to achieve this goal is to perform spectroscopic studies on Co(II)-substituted enzyme and compare the spectra of the enzyme in the absence and presence of a competitive inhibitor. Previously, Page and co-workers reported that MEPA is a competitive inhibitor of β-lactamase II and argued that MEPA structurally resembled a portion of the β-lactam-containing substrates (20). Since previous inhibition studies have revealed that the metallo-β-lactamases exhibit widely differing efficacies and modes of inhibition toward small molecule inhibitors (19, 24, 25), we synthesized MEPA and tested its inhibition properties toward L1. As with β-lactamase II, MEPA is a competitive inhibitor of L1 with a K_i of 50 ± 3 μM.

The crystal structure and sequence of L1 revealed that L1 contains two cysteine residues (Cys218 and Cys246), and these cysteines form a disulfide bridge which was proposed to constrain a large loop between α helix 5 and β sheet 12 (15). Since MEPA has a free thiol group (Fig. 1), we believed that the inhibitor could disrupt the disulfide bridge between Cys218 and Cys246 and possibly offer insight into the role of this loop; therefore, inactivation studies were performed in an attempt to observe a slow, time-dependent inactivation of L1. The results of these inactivation studies revealed that no such inactivation occurs when L1 and MEPA are reacted.

These studies were consistent with MEPA being a reversible, competitive inhibitor of L1, and it could be used for future spectroscopic and crystallographic studies to probe L1 structure upon inhibitor/substrate binding; however, the binding of MEPA to L1 is relatively weak and would require high concentrations of inhibitor (up to 530 μM or 10× K_i) to saturate L1 in such studies. We, therefore, hoped to discover another competitive inhibitor that more tightly binds to L1.

Previously, Payne et al. reported that a series of mercaptoacetic acid thiol esters were inhibitors of L1, β-lactamase II from B. cereus, CfiA from B. fragilis, and CpaA from A. hydrophila (19, 25). These thioesters exhibited greatly differing inhibitory potencies and modes of inhibition toward the tested metallo-β-lactamases; in fact, several of the compounds irreversibly inactivated some of the metallo-β-lactamases (19, 25). A general trend in inhibition was that added Zn(II) in the activity assays protected β-lactamase II from inhibition by these compounds; however, Zn(II) had little or no effect on the IC_50's of these compounds toward L1 (19). Further study using β-lactamase II and three of the thioesters demonstrated that the compounds inhibited β-lactamase II by a mechanism-based delivery of mercaptoacetic acid to the Zn(II)-binding cysteine. The added Zn(II) in activity assays probably protected the active-site cysteine from mercaptoacetate binding because the cysteine was bound to Zn(II). In the case of L1, however, there is no metal binding cysteine, and therefore, the mode of inhibition of L1 was uncertain.

Of the reported thioesters, BATA (SB214752) was reported to inhibit L1 with an IC_50 of 2–3 μM in the presence or absence of added Zn(II) (19). We synthesized BATA and used steady-state inhibition studies to determine that the compound is a competitive inhibitor of L1 with a K_i of 1.6 ± 0.3 μM. Since Payne et al. had reported that mercaptoacetic acid is hydrolyzed from the mercaptoacetic acid thioesters (19), we wanted to know if mercaptoacetate was covalently attaching to L1 after incubation with the thioester. MALDI–TOF mass spectrometric studies demonstrated two equivalents of mercaptoacetate (M, 90 g/mol) were added to L1 after incubation of L1 with BATA. Since there are only two cysteines in L1 (Cys218 and Cys246) (15, 26), these are the most likely sites for mercaptoacetate attachment. These mercaptoacetates are probably covalently attached to L1 since the MALDI process with α-cyano-4-hydroxycinnamic acid as a matrix typically does not allow for the detection of noncovalently bound complexes (M. W. Crowder, unpublished) (27–29). Under the conditions described under Experimental Procedures, only 30–40% of the L1 was covalently modified as judged by the relative peak intensities in Fig. 3B. However, for Cys218 and Cys246 to be modified, the mercaptoacetates must diffuse from the active site where the inhibitor is hydrolyzed to the loop for covalent bond formation. This diffusion-related event certainly reduces the efficiency of covalent modification.

To determine whether the attachment of mercaptoacetates to L1 affects catalytic activity, we incubated L1 with BATA under the same conditions used for the MALDI experiment above and then dialyzed the reaction mixture to remove excess inhibitor. Activity assays demonstrated that there was no loss in activity in the mercaptoacetate-modified L1, suggesting that the
disulfide bridge is not necessary for catalysis and that the loop containing the cysteines may not play a significant role in L1 catalysis.

Recently, Fitzgerald et al. reported that Mes is a weak competitive inhibitor of CcrA with a $K_i$ of $23 \pm 5$ mM (17). These researchers published a crystal structure of CcrA with Mes bound in the active site, and this study implicated several amino residues and a small “clamp” loop as being important for CcrA catalysis. L1 also has a similar clamp loop, and Ullah et al. implicated several amino acid residues on that loop as being important in enzyme–substrate contacts (15). In an attempt to determine if Mes binds to L1, inhibition studies using Mes as the inhibitor were conducted. However, Mes did not inhibit nitrocefin hydrolysis by L1, even at concentrations of up to 300 mM.

The results of this work further highlight the structural and inhibition differences of the metallo-$\beta$-lactamases. The structural and mechanistic heterogeneity of the metallo-$\beta$-lactamases suggests that one inhibitor may not be a clinically useful inhibitor for all enzymes. This concern necessitates a detailed characterization of at least one metallo-$\beta$-lactamase from each of the subgroups of the group 3 $\beta$-lactamases (4, 13, 14). Only after this detailed characterization can real similarities between the metallo-$\beta$-lactamases be identified and exploited for the preparation of inhibitors. The work presented here has identified two useful competitive inhibitors for structural studies on L1, and these studies are currently in progress.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health (R29 AI40052), and funding for the purchase of the mass spectrometer was provided by the National Science Foundation (CHE-9413529) and the Ohio Board of Regents (Obor) Action Fund. The authors thank Dr. Ying Yang for her expert assistance in the acquisition of the MALDI–TOF mass spectra and Dr. Timothy Walsh for helpful discussions.

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