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ARTICLE

Thiosemicarbazones exhibit inhibitory efficacy against New Delhi metallo-β-lactamase-1 (NDM-1)

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Abstract

The superbug infection caused by metallo-β-lactamases (MβLs) carrying drug-resistant bacteria, specifically, New Delhi metallo-β-lactamase (NDM-1) has become an emerging threat. In an effort to develop novel inhibitors of NDM-1, thirteen thiosemicarbazones (**1a**-**1m**) were synthesized and assayed. The obtained molecules specifically inhibited NDM-1, with an IC_{50} in the range of 0.88–20.2 μM, and **1a** and **1f** were found to be the potent inhibitors ($IC_{50} = 1.79$ and 0.88 μM) using cefazolin as substrate. ITC and kinetic assays indicated that **1a** irreversibly and non-competitively inhibited NDM-1 in vitro. Importantly, MIC assays revealed that these molecules by themselves can sterilize NDM-producing clinical isolates EC01 and EC08, exhibited 78-312-fold stronger activities than the cefazolin. MIC assays suggest that **1a** (16 μg ml^{-1}) has synergistic antimicrobial effect with ampicillin, cefazolin and meropenem on *E. coli* producing NDM-1, resulting in MICs of 4-32-, 4-32-, and 4-8-fold decrease, respectively. These studies indicate that the thiosemicarbazide is a valuable scaffold for the development of inhibitors of NDM-1 and NDM-1 carrying drug-resistant bacteria.

Introduction

Since the discovery of penicillin in the last century, β-lactams, as the most effective and successful antibiotics, have been widely used for the treatment of bacterial infections [1]. However, the overuse of β-lactam antibiotics has resulted in a large number of bacteria that produce β-lactamases, and the resulting bacteria are resistant to the most commonly-used antibiotics, including penicillins, cephalosporins and carbapenems. Commonly, bacteria become resistant to β-lactam antibiotics by producing β-lactamases [2]. β-Lactamases are enzymes that inactivate β-lactam antibiotics by breaking the C-N bond of β-lactam ring and render the drugs ineffective [3].

So far, there have been more than 2800 distinct β-lactamases identified [4], and these enzymes have been categorized into classes A, B, C and D, based on their amino acid sequence homologies and action mechanism. Classes A, C and D enzymes are referred to as serine-β-lactamases (SβLs), which utilize an active site serine to nucleophilically attack the β-lactam carbonyl, ultimately leading to a cleaved β-lactam ring. Class B enzymes, also called metallo-β-lactamases (MβLs), use either 1 or 2 equivalents Zn(II) to catalyze β-lactams hydrolysis. MβLs are further divided into subclasses B1, B2, and B3, based on their amino acid sequence homologies and Zn(II) content [5]. MβLs belonging to the B1 and B3 subclasses can hydrolyze almost all known β-lactam antibiotics. In contrast, the B2 subclass enzymes have a narrow substrate profile including carbapenems, which have been called one of the "last resort" antibiotics [6]. There are no known clinical inhibitors of the MβLs to date [7].

Given the enormous biomedical importance of MβLs, a large amount of effort have been made to develop inhibitors

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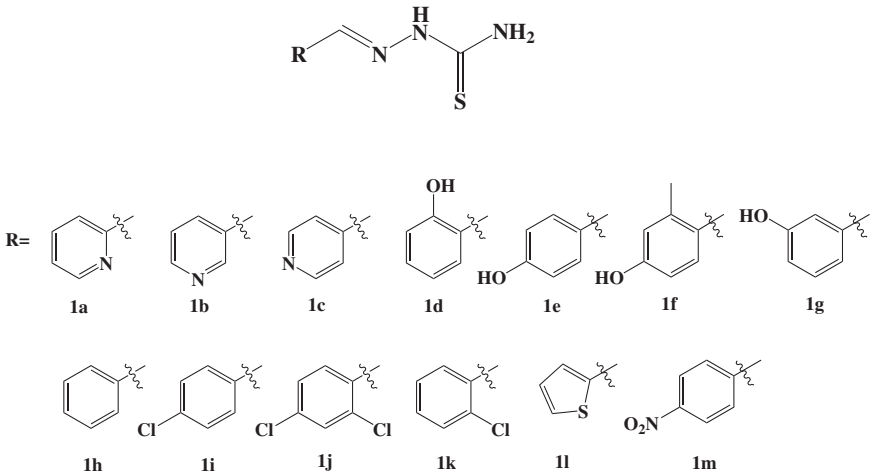

Fig. 1 Structures of the synthetic thiosemicarbazones

of the enzymes [8], such as azolylthioacetamides [9], bis-thiazolidines [10], and maleic acids [11], which exhibit inhibition effect by binding to Zn(II) of the enzymes; chelating inhibitors, represented as aspergillomarasmine A (AMA) [12], and [*S*,*S*]-ethylenediamine-*N*,*N*'-disuccinic acid (EDDS) [13], which inactivate MβLs like VIM-2 and NDM-1 by sequestering the metals of each enzyme; and covalent inhibitors, such as ebselen, which inactivate the B1 and B2 subclass MβLs by formation of a Se-S bond with a cysteine residue at active site of enzymes [14]. Recently, our studies revealed that the azolylthioacetamide is a highly promising scaffold for the development of MβLs inhibitors with IC_{50} values in submicromolar range [15].

New Delhi metallo-β-lactamase-1 (NDM-1), a B1 subclass MβL, hydrolyzes almost all β-lactam antibiotics including carbapenems [16]. The plasmid-encoded NDM-1 gene has been shown to horizontally transfer to other pathogenic bacteria, and therefore led to multiple drug resistance and caused spread of the resistant bacteria around the world [17]. Given the enormous importance of NDM-1, a large number of NDM-1 inhibitors have been reported, such as metal complexing reagent [18], binary carboxylic acid [19], D- and L-captopril [20], and ebuprofen derivative [21]. Recently, ebselen has been reported to be the covalent inhibitor of NDM-1 by forming a S–Se bond with the Cys221 residue at active site of the enzyme [3, 22].

Thiosemicarbazone, an inhibitor of urease and tyrosinase, has been widely used for antitumor and antibacterial studies [23, 24].

The urease and tyrosinase, both dizinc-containing enzymes like B1 and B3 subclasses MβLs, reminded us of that the thiosemicarbazone may be a potential scaffold for the development of MβLs, specifically NDM-1 inhibitors. In addition, Spyrakis, et al, reported that the compounds bearing a thiol, a thiosemicarbazide or thiosemicarbazone moiety had a good inhibitory effect on NDM-1 [25]. Our goal is to develop specific inhibitors of MβLs and to use these inhibitors in combination with β-lactam antibiotics to combat bacterial infections. Towards this goal, the thiosemicarbazones with various aromatic substituents (Fig. 1) were synthesized and characterized by NMR and MS. These compounds were tested as inhibitors with the purified enzyme NDM-1, their inhibitory mode was investigated by generating Lineweaver–Burk plots and isothermal titration calorimetry (ITC). Also, the antimicrobial activities of these inhibitors were evaluated in combination with the existing antibiotics against antibiotic-resistant strains.

Materials and methods

General Information

General chemicals were purchased from TCI (Tokyo Chemical Industry, Tokyo, Japan), Sigma-Aldrich (US), Energy-Chemical (China) and Aladdin (China). The instruments used in experiments include TOF-Q mass spectrometer, agilent-8453 UV-Vis spectrophotometer, AKTA purifier 10, FD-1C freeze dryer and IKA magnetic stirrer. The plasmid of NDM-1 and *Escherichia coli* BL21 cells were donated by Professor Michael Crowder at Miami University, USA. The plasmid of NDM-1 was transformed into BL21 *E. coli* to offer the engineering bacteria *E. coli*-NDM-1 that only produce NDM-1 enzyme for protein expression and MIC evaluation. Analytical data for all synthesized compounds can be found in the supplementary file. The clinical strains *E. coli* harboring NDMs (EC01, EC08) were obtained from the First Affiliated Hospital of Xi'an Jiaotong University.

Inhibition studies

To test whether these thiosemicarbazones were NDM-1 inhibitors, the inhibition experiments under steady-state

conditions were conducted on an Agilent UV8453 spectrometer using cefazolin as substrate. The thiosemicarbazones, NDM-1 and substrates samples were prepared with 30 mM Tris, pH 7.0. The final enzyme concentration was 13 nM and the concentration of cefazolin was 50 μM. The concentrations of inhibitors were varied between 0 and 20 μM. Enzyme and inhibitor were pre-incubated for 20 min before adding substrate. Hydrolysis of cefazolin was monitored at 265 nm. The initial reaction rates were determined in the absence and presence of inhibitors in triplicate, and the average values were recorded. The IC_{50} values were linear fitted by plotting the average percentage inhibition against inhibitor concentration.

To further identify the inhibition mode of the thiosemicarbazones on NDM-1, **1a** was chosen to determine K_i values. Concentrations of the inhibitor were varied between 0 and 10 μM, and substrate (cefazolin) concentrations were varied between 20 and 100 μM. All experimental hydrolytic rates were determined in triplicate. The inhibition mode was assayed by generating Lineweaver–Burk plots, and K_i values were obtained by fitting the initial velocity versus substrate concentration at each inhibitor concentration using SigmaPlot 12.0.

Isothermal titration calorimetry (ITC) assays

Isothermal titration calorimetry (ITC), an essential approach to measure the heat (Q) released or absorbed during reaction by SigmaPlot been applied in enzyme kinetic studies [26]. A MicroCal-ITC200 was employed to investigate the inhibition of cefazolin hydrolysis with NDM-1 by thiosemicarbazone in single injection mode at 25 °C as previously reported [27]. The concentrations of the enzyme and cefazolin were 100 nM and 0.1 mM, respectively, and the concentrations of inhibitor were varied between 0 and 120 μM.

Determination of MIC

The MIC values of the antibiotics alone and in the presence of the enzyme inhibitor against antibiotic-resistant bacteria were determined using the broth micro-dilution method. Single colonies of cells were transferred into 5 ml of Mueller–Hinton (MH) liquid medium. Strains, grown in MH medium to $OD_{600} = 0.45$, were used as inocula after 84-fold dilution to 1×10^5 CFU ml^{-1} in MH medium. Antibiotics and inhibitors were dissolved in MH medium to prepare different concentration stock solutions. These solutions, with different antibiotic concentrations (50 μl), were diluted to 100 μl with 50 μl inhibitor solution, then 100 μl inoculum was added, sequentially, into the prepared solutions. The mixtures were incubated at 37 °C for 16 h and the minimum concentration of completely inhibited bacteria was MIC value. Each measurement was repeated three times.

Results and discussion

Synthesis of thiosemicarbazones

Thirteen thiosemicarbazones **1a**-**1m** (Fig. 1) were synthesized by previously reported methods [28]. Briefly, the aromatic aldehyde and thiosemicarbazide were refluxed in ethanol with 0.1 equivalent pyrrolidine as catalyst for 5 h, and the reaction mixture was gradually cooled to room temperature, filtrated, washed with cold ethanol, and dried to give the product thiosemicarbazones, with a yield of more than 90%.

Inhibitory activity assay

Percent inhibition, defined as enzyme activity without inhibitor (100%) minus residual activity with inhibitor, of thiosemicarbazones on NDM-1 is shown in Fig. S1. The results show that all compounds exhibited more than 34% inhibition against NDM-1 at a concentration of 10 μM, especially **1a** and **1f** showed more than 86% inhibition.

The inhibitor concentrations causing 50% decrease of enzyme activity (IC_{50}) of all thiosemicarbazones against NDM-1 were determined in 30 mM Tris (pH 7.0) using cefazolin as substrate. These kinetic experiments were done in triplicate, and the average values±standard deviations are reported in Table 1. The collected IC_{50} data indicated that all of these compounds had inhibitory efficacy on NDM-1, exhibiting an IC_{50} value in the range of 0.88–20.2 μM, and **1a** and **1f** were found to be the most potent inhibitors (IC_{50} = 1.79 and 0.88 μM). The structure-activity relationship analysis reveals that the thiosemicarbazones with nitrogen heterocyclic ring or hydroxyl aromatic ring, particularly *p*-hydroxyl aromatic ring has the better inhibition activity than the molecules with other substitutes. Given the good potency of thiosemicarbazone **1a**, the time- and concentration-dependent inhibitions of it on NDM-1 were assayed (Fig. S2). NDM-1 and **1a**

Table 1 IC_{50} values of thiosemicarbazones against NDM-1

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
1a	1.79 ± 0.01	**1h**	16.7 ± 0.04
1b	6.81 ± 0.02	**1i**	19.8 ± 0.03
1c	8.19 ± 0.02	**1j**	15.7 ± 0.02
1d	6.98 ± 0.03	**1k**	20.2 ± 0.02
1e	9.11 ± 0.02	**1l**	9.87 ± 0.02
1f	0.88 ± 0.01	**1m**	17.4 ± 0.03
1g	7.49 ± 0.03		

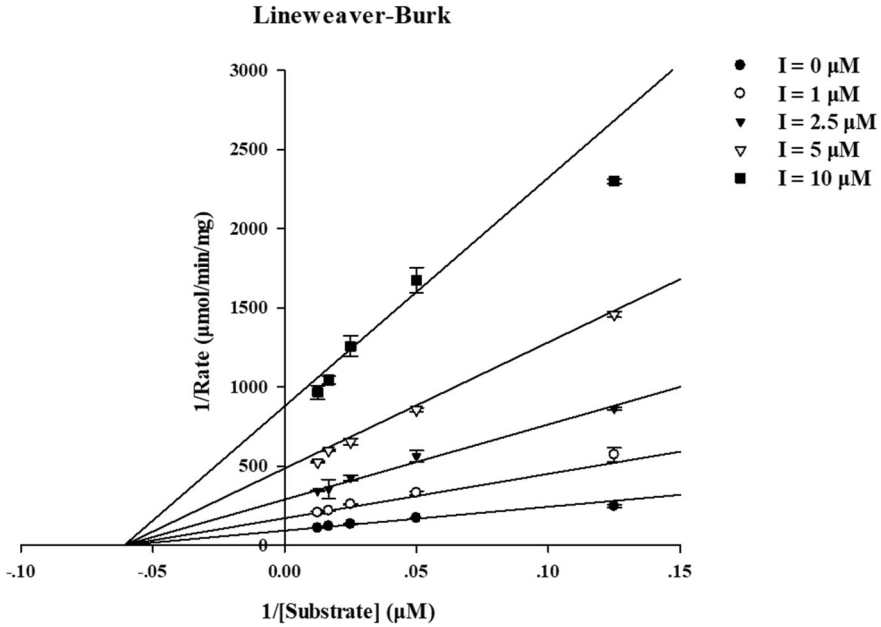

Fig. 2 Lineweaver–Burk plots of cefazolin hydrolysis catalyzed by NDM-1 in the absence and presence of thiosemicarbazone **1a**. Inhibitor concentration was in the range of 0–10 µM

were incubated for different time before adding cefazolin. It can be observed that, in the presence of **1a** at a concentration of 10 µM, the residual activity of NDM-1 decreased with the extension of preincubation time, and the molecule showed the maximum inhibition after incubation with the enzyme for about 80 min (Fig. S2A), indicating that the compound is a time-dependent inhibitor. Also, it is observed (Fig. S2B) that the percent inhibition of **1a** on NDM-1 increased with the increase of inhibitor concentration. The Lineweaver–Burk plots of cefazolin hydrolysis catalyzed by NDM-1 in the absence and presence of thiosemicarbazone (Fig. 2) suggest that 1a is a non-competitive inhibitor, and the Ki value determined is 1.2 µM, which is lower than the data (10.7 µM) of thiosemicarbazone moiety reported by Spyrakis, et al [25].

Inhibition assay by ITC

Monitoring of the inhibition of imipenem hydrolysis with NDM-1 by **1a** is shown in Fig. 3. It is clearly observed that the thermopower (dq/dt) gradually decreased with the increase in the inhibitor concentrations from 0 to 120 µM, confirming that **1a** is a dose-dependent inhibitor of NDM-1 thermodynamically. However, the addition of the compound resulted in the decrease of total heat (Q) released, revealing that the thiosemicarbazone irreversibly inhibited NDM-1, similar to the inhibition mode of ebselen on NDM-1, as previously reported [27]. The Q is an important indicator to discriminate the mode of action of inhibitors. When Q remains constant, the inhibitor is a reversible inhibitor, but the inhibitor molecule that reduces Q is an irreversible inhibitor [27]. This irreversible inhibition is probably due to the binding of **1a** to two zinc ions at active site of NDM-1.

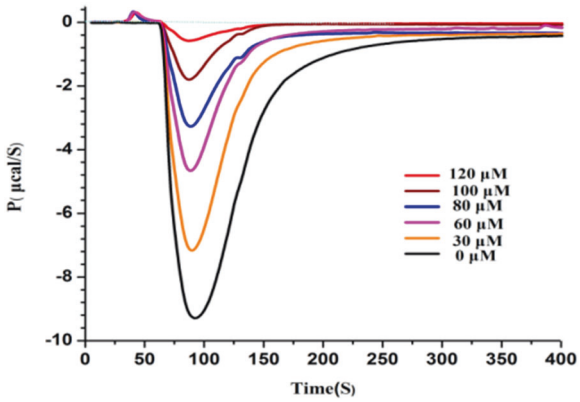

Fig. 3 Overlayed heat flow curves of NDM-1 enzyme catalyzed hydrolysis of cefazolin in the absence and presence of thiosemicarbazone **1a** at a concentration in the range of 0–120 µM, using a single injection mode ITC assay at 25 °C

MIC determination

The sterilizing ability and synergistic antimicrobial activity with antibiotic of thiosemicarbazones were investigated by determining the minimum inhibitory concentrations (MICs) in the absence and presence of **1a**-**1m** as previously reported [29]. The drug-resistant strains BL21 *E. coli* producing NDM-1 (*E. coli*-NDM-1), and clinical isolates *E. coli* harboring NDMs (EC01, EC08) were used to assay the inhibitors, and the antibiotics used were cefazolin, ampicillin, and meropenem. The collected MIC data (Table 2a) indicated that all of the tested thiosemicarbazides alone exhibited antimicrobial effect against EC01 and EC08, with a MIC value that is 4-312-fold lower than that of cefazolin, but **1a**, **1d**, **1f**,

Table 2 Antibacterial activities (MICs, μg ml^{-1}) of thiosemicarbazones **1a**-**1m** against three drug-resistant strains expressing β-lactamases (a), and MICs of **1a** and **1f** (16 μg ml^{-1}) synergize three antibiotics (b)

a

compd.	*E.coli*-NDM-1	EC01	EC08	compd.	*E.coli*-NDM-1	EC01	ECO8
1a	64	64	64	**1h**	1024	1024	1024
1b	256	256	256	**1i**	256	256	256
1c	256	128	256	**1j**	64	64	64
1d	128	128	128	**1k**	32	64	32
1e	256	256	256	**1l**	256	256	256
1f	32	64	32	**1m**	32	128	64
1g	256	256	256	cefazolin	256	20000	5000

b

Strains	Ampicillin	Cefazolin	Meropenem
E.coli-NDM-1			
Antibiotic	512	256	128
+**1a**	32	8	16
+**1f**	16	4	8
EC08			
Antibiotic	20,000	5000	150
+**1a**	5000	1250	37.5
+**1f**	2500	625	75

1j-**1k**, and **1m** had a MIC that is 4-8-fold lower than the antibiotic against BL21 *E. coli*. The MIC data (Table 2b) showed that the thiosemicarbazides **1a** and **1f** tested (16 μg ml^{-1}) increased the antimicrobial effect of ampicillin, cefazolin and meropenem against *E. coli*-NDM-1 and EC08, and resulted in decreased MICs for ampicillin 4-32-fold, cefazolin 4-64-fold, and meropenem 4-16-fold, respectively. Analysis of MIC data of **1a** on *E.coli*-NDM-1 suggests that the compound has dual functions on *E.coli*-NDM-1, that is, enzyme inhibition and bacteriostatic efficacy.

Conclusion

Thirteen thiosemicarbazones (**1a**-**1m**) were synthesized and characterized by ^{1}H and ^{13}C NMR and MS. Biological assays revealed that these compounds inhibited NDM-1, with an IC_{50} value in the range of 0.88-20.2 μM, and **1a** and **1f** were found to be the bestinhibitors (IC_{50} = 1.79 and 0.88 μM). ITC characterization indicated that **1a** irreversibly inhibited NDM-1 in vitro, and the identification of K_i showed **1a** to be a non-competitive inhibitor. MIC assays revealed that these molecules alone can inhibit the growth of NDM-producing isolates EC01 and EC08, exhibited 4-312-fold stronger activities than the cefazolin, and **1a** and **1f** (16 μg ml^{-1}) tested shown synergistic antimicrobial effect with ampicillin, cefazolin and meropenem on *E. coli* producing NDM-1 and EC08, resulting in MICs of 4-32-, 4-64-, and 4-16-fold decrease, respectively.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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