Small-molecule inhibitors of bacterial metallo-β-lactamases

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Chapter 4

Thiol-containing metallo-β-lactamase inhibitors: synergy, zinc-binding affinity and stability evaluation.

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1. Introduction

Resistance to β-lactam antibiotics poses a serious threat to human health. The enzymes responsible for such resistance are the β-lactamases and are especially prevalent among gram-negative bacteria.[1,2] These enzymes are divided in two classes: the serine β-lactamases (SBLs) which hydrolyze β-lactam ring by a serine nucleophile in their active site; and the metallo-β-lactamases (MBLs) whose mechanism relies upon the presence of one or two active site zinc ions.[3] These zinc ions stabilize a nucleophilic hydroxide species that is believed to be the active agent in the hydrolysis the β-lactam ring leading to antibiotic inactivation. The best-studied MBLs include the NDM (New Delhi metallo-β-lactamase), VIM (Verona integron-encoded metallo-β-lactamase) and IMP (imipenemase) enzymes which collectively exhibit a broad substrate specificity and hydrolyze antibiotics from all known β-lactam classes with the exception of monobactams.[4]

The global concern relating to antibiotic resistance has led to a number of different strategies aimed at addressing the problem. One such approach involves the co-administration of antibiotic adjuvants capable of maintaining the activity of existing antibiotics.[5] This strategy is widely effective in treating infections due to bacteria that express SBLs. Clinically relevant SBL inhibitors include clavulanic acid, sulbactam, tazobactam, avibactam, and vaborbactam which effectively protect β-lactam antibiotics from inactivation when administered as combination therapies. By comparison, there are no clinically used MBL inhibitors available for use in addressing the growing threat posed to the β-lactam arsenal by these enzymes.

Attempts to identify inhibitors of the MBLs have revealed a number of compound classes that display promising activities when tested using *in vitro* enzyme inhibition assays.[6] Such compounds include small molecules that contain functionalities often associated with zinc binding such as thiols, dicarboxylates, hydroxamates, aryl sulfonamides, *N*-arylsulfonyl hydrazones and tetrazole-based compounds.[3,7,8] Among the known MBL inhibitors, sulfur-containing small molecules containing either a free thiol or a sulfur atom masked as a heterocycle[9,10] are among the best characterized.[3,7,11–15] Structures as simple as mercaptoacetic acid and mercaptopropionic acid have been shown to be effective inhibitors of the IMP-1 enzyme (K_i = 0.23 μM and 0.19 μM respectively).[16] Follow-up studies identified higher analogs of mercapto-carboxylic acids including 2-arylmethyl-2-mercaptoacetic acids and their thioesters as potent MBL inhibitors with IC_{50} values in the low-nanomolar range.[17] Similarly, thiomandelic acid was found to be a broad-spectrum inhibitor of different MBLs including BCII (K_i = 0.34 μM),

IMP-1 (K_i = 0.029 μM), IMP-2 (K_i = 0.059 μM) and VIM-1 (K_i = 0.230 μM)[18], and 2-ω-phenylpropyl-3-mercaptopropionic acid has been reported as potent inhibitor of VIM-2 (K_i = 0.220 μM).[19,20] Other examples of structurally similar MBL inhibitors include compounds containing a free thiol with a neighboring carbonyl functionality such as α-mercaptoacetophenone,[21] thiosalicylic acid,[18] and interestingly, the dipeptide drug captopril which is used in the treatment of hypertension.[22] The inhibitory activity of these molecules is attributed to the ability of the thiol group to bind the zinc ion present in the MBL active site as supported by several X-ray crystallography studies.[21,23–26]

The inhibitory activity of thiol-containing small molecules against clinically relevant MBLs *in vitro* prompted us to conduct a series of antibacterial assays to evaluate the synergistic activity of such compounds with the representative β-lactams meropenem and cefoperazone. To do so, the sensitizing effects of thiols **1**-**5** (figure 1) on the activity of meropenem and cefoperazone were assessed against a panel of gram-negative bacteria expressing various β-lactamases. The stability of the thiols was also assessed under the assay condition employed and isothermal titration calorimetry (ITC) was used to measure the zinc-binding affinity of the most synergistically-active compounds.

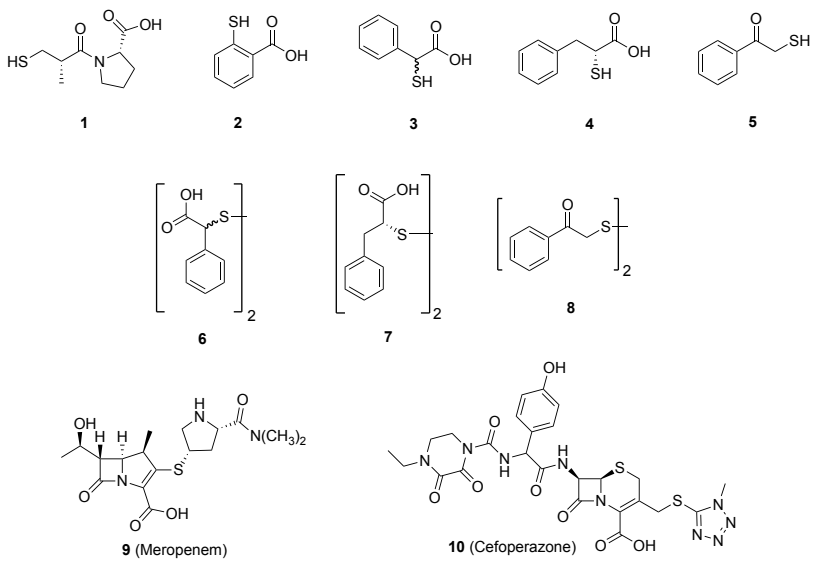


Figure 1. Thiol-based MBL inhibitors and disulfides evaluated for synergy with meropenem and cefoperazone in the current study.

2. Results and discussion

Thiols **1**-**5** were initially tested alone for antibacterial activity against a panel of carbapenem-resistant gram-negative pathogens expressing MBLs including NDM, VIM and IMP enzymes or SBLs such as KPC-2 and OXA-48. These studies revealed that none of the thiols inhibited bacterial growth at the highest concentration tested (64 μg/mL). All of the MBL-expressing strains used in our study exhibited resistance to both meropenem and cefoperazone with MIC values ranging from 8 to >256 μg/mL. For use as a reference MBL inhibitor known to synergize with β-lactam antibiotics, we turned to the work of Migliavacca and coworkers who reported a zinc chelating mixture of EDTA and 1,10-phenanthroline as being synergistic with imipenem in preventing growth of MBL-expressing strains of *Pseudomonas aeruginosa*.[27] We found that the EDTA/1,10-phenanthroline mixture was similarly effective in lowering the MIC of meropenem and cefoperazone against the MBL-expressing strains used in our study (table 1). Also, and as expected, the EDTA/1,10-phenanthroline mixture showed no synergistic effect with meropenem against the two SBL-expressing strains also evaluated.

With a reliable reference system in hand, the capacity for thiols **1**-**5** to synergize with meropenem was next investigated. Table 1 shows the synergy data of thiols **1**-**5**. Captopril **1** exhibited moderate to weak synergy at a concentration of 64 μg/mL while thiosalicylic acid **2** displayed no appreciable synergy when tested at the same concentration. By comparison, when administered at 64 μg/mL thiols **3** and **4** significantly lowered the MIC of meropenem against all the MBL-producing isolates tested. The activity of compound **5** was also promising but interestingly limited to only the two *Klebsiella* strains tested. Thiols **3**-**5** have previously been shown to be more potent MBL inhibitors than compounds **1** and **2** in biochemical enzyme inhibition assays[17,18,21,23] and our MIC synergy results follow the same trend. Notably, for compounds **3** and **4** we observed broad-spectrum, and in some cases, potent synergistic activity with meropenem against the MBL-producing isolates evaluated.

Table 1. MIC of meropenem (Mer) and Ceferazone (Cef) tested alone or in combination with thiol MBL-inhibitors **1**-**5**

Isolates	Mer	Cef	Mer + **1**[a]	Mer + **2**	Mer + **3**	Cef + **3**	Mer + **4**	Cef + **4**	Mer + **5**	EPM[b]	EPC[b]
K. pneumoniae (KPC-2)	>128	>256	>128	>128	128	>256	>128	>256	128	>128[c]	>256[c]
K. pneumoniae (OXA-48)	64	>256	64	64	64	>256	64	>256	64	16[d]	>256[d]
K. pneumoniae (VIM-1)	64/32	>256	8 (4)[e]	16 (2)	0.5 (≥128)	256	1 (32)	>256	8 (8)	≤0.5[c]	8[c]
K. pneumoniae (IMP-28)	16/8	256	1 (8)	8	0.125 (128)	≤2 (≥128)	0.125 (64)	≤2 (≥128)	0.5 (32)	≤0.125[f]	≤2[f]
E. coli (NDM-1)	128/64	>256	16 (4)	128	16 (8)	>256	16 (8)	>256	64 (2)	≤1[c]	>256[c]
P. aeruginosa (VIM-2)	32	128	32	32	4 (8)	16 (8)	16 (4)	16 (8)	32	0.5[c]	8[c]
P. aeruginosa (IMP-13, IMP-37)	64	256	64	64	8 (8)	8 (32)	8 (8)	16 (16)	64	4[f]	4[f]
K. pneumoniae (VIM-1)	64/32	>256	n.d.	n.d.	0.5 (64)	>256	1 (32)	>256	n.d.	≤0.5[f]	≤2[f]
E. aerogenes (VIM-1)	32/16	>256	n.d.	n.d.	0.5 (64)	>256	1 (16)	>256	n.d.	≤0.25[f]	64[f]
K. pneumoniae (VIM-1)	>128/128	>256	n.d.	n.d.	4 (>32)	>256	8 (16)	>256	n.d.	≤1[f]	≤2[f]
K. pneumoniae (NDM-1)	32/16	>256	n.d.	n.d.	8 (4)	>256	2 (16)	>256	n.d.	≤0.5[f]	>256[f]
K. pneumoniae (NDM-1)	16/8	>256	n.d.	n.d.	4 (4)	>256	1 (8)	>256	n.d.	≤0.25[f]	>256[f]
K. pneumoniae (NDM-1)	64/32	>256	n.d.	n.d.	16 (4)	>256	16 (4)	>256	n.d.	≤1[f]	>256[f]

[a]Thiols **1**-**5** added at 64 μg/mL
[b]EPM: EDTA/phenanthroline/meropenem; EPC: EDTA/phenanthroline/cefoperazone
[c]EP mixture used at 16 and 1 μg/mL respectively
[d]EP mixture used at 64 and 4 μg/mL respectively
[e]Fold reduction of MIC shown in parentheses
[f]EP mixture used at 32 μg/mL and 2 μg/mL respectively

Building on the encouraging results of the preliminary synergy assays (carried out at fixed thiol concentration of 64 μg/mL) we next performed a series of checkerboard synergy assays in which the MIC of meropenem was determined at varying concentrations of inhibitors **1**-**5**. Such an approach provides for a better picture of the synergistic relationship between the two combined agents and allows for determination of the fractional inhibitory concentration (FIC) index. Briefly, FIC values are calculated by adding the following two fractional values: (MIC of compound A in combination/MIC of compound A alone) + (MIC of compound B in combination/MIC of compound B alone). In general, an FIC index value <0.5 is regarded as an indication of synergy.[28] Among the MBL-expressing strains used, the two *Klebsiella* isolates were most effectively resensitized to meropenem when administered in combination with thiols **3**-**5**. Of particular note, compounds **3** and **4** were both found to significantly potentiate meropenem against the IMP-28 producing *Klebsiella* strain tested with FIC values ≤0.07 and ≤0.13 respectively.

Thiols are well known for their tendency to form homo- or heterodisulfides in biological systems. Such reactivity is of special importance in the case of thiol-based MBL inhibitors such as compounds **1**-**5** as it has been reported that in their disulfide form their activity is significantly reduced.[18] In this regard we selected compounds **3**-**5** as the three most active thiols from our synergy assays and monitored their conversion to the corresponding disulfides under the assay conditions used. Thiols **3**-**5** were thus incubated in Mueller-Hinton broth at 37 °C and sample aliquots analyzed at time points ranging from 0 to 8 hours. As shown in figure 2, thiols **3** and **4** were found to form their corresponding disulfides (**6** and **7** respectively) with half-lives of *ca*. 5 hours. By comparison, thiol **5** was oxidized to **8** more rapidly with a half-life in the range of minutes which may also explain its lower level of synergy relative to **3** and **4**. Disulfides **6**-**8** were synthesized for use as reference compounds in the stability assays and were evaluated for their synergy with meropenem against the two most susceptible *Klebsiella* isolates identified (table 2). The three disulfides exhibited very low levels of synergy relative to that of the corresponding free thiols. The slight synergy observed for these disulfides may in fact be attributable to a reductive process carried out by the bacteria themselves to release a small amount of the more active thiol. Many bacteria contain redox active enzymes capable of disulfide reduction both in cytoplasm and periplasmic space.[29]

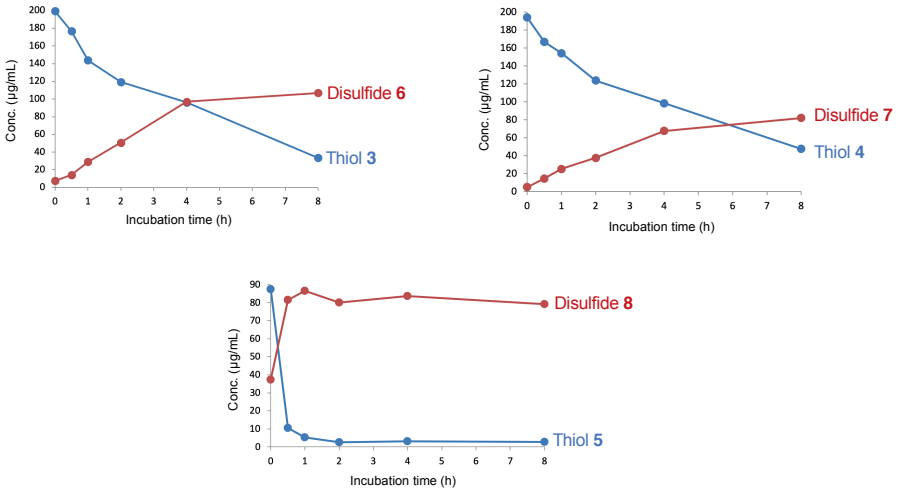


Figure 2. Time-dependent oxidation of thiols **3**-**5** to corresponding disulfides **6**-**8** by incubation in Mueller-Hinton broth at 37 °C.

The superior synergistic activity and relative stability of thiols **3** and **4** prompted us to further characterize their zinc binding abilities using isothermal titration calorimetry (ITC). To do so a solution of zinc chloride was titrated into the sample well containing either **3** or **4** (both found to be stable in the buffer conditions used for the ITC experiments) and the heat of binding monitored. In this way a number of thermodynamic binding parameters are revealed including K_d (dissociation constant), ΔH (enthalpy), ΔG (Gibbs free energy) and ΔS (entropy). As shown in figure 3, compounds **3** and **4** exhibited high affinities for Zn^{2+} with K_d values of 9.8 and 20.0 µM respectively. Also of note was the lack of any measurable binding interaction when zinc chloride was titrated into solutions of disulfides **6** and **7**. The zinc binding abilities of the reference compounds EDTA and 1,10-phenanthroline were also assessed using ITC showing strong

Table 2. MIC of meropenem (Mer) tested alone or in combination with disulfides **6**-**8**

Isolates	Mer	Mer + **6**[a]	Mer + **7**	Mer + **8**
K. pneumoniae (VIM-1)	64	64	64	32 (2)[b]
K. pneumoniae (IMP-28)	8	1 (8)	4 (2)	1 (8)

[a]Disulfides **6** and **7** were added at 64 µg/mL and **8** added at 32 µg/mL.
[b]Fold reduction of MIC has been shown in parentheses

interactions with K_d values of <100 nM and 2.3 μM respectively (see experimental section for thermograms). The results of these ITC studies correlate well with the synergy data obtained and suggest that zinc binding may be a useful predictor for a compound's ability to resensitize MBL-expressing organisms to β-lactam antibiotics. Furthermore, the relative ease with which ITC can be used to assess zinc binding by small molecules may make it a complimentary technique for identifying new lead compounds capable of effectively inhibiting MBLs.

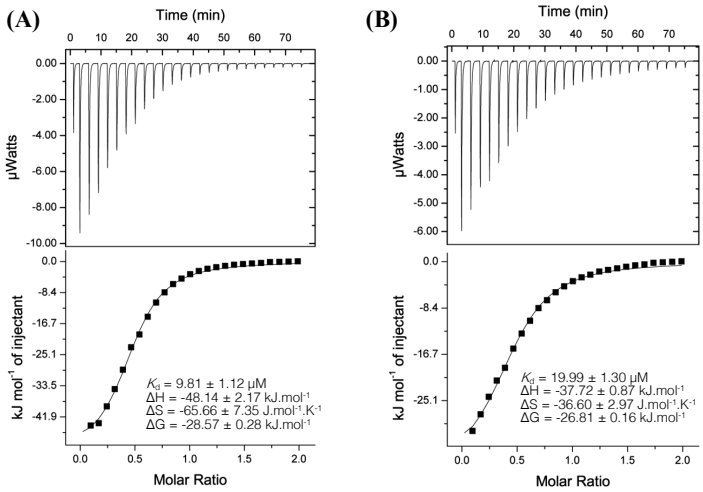


Figure 3. ITC Thermograms for binding of Zn^{2+} by thiols **3** (A) and **4** (B). A solution of zinc chloride (2.0 mM) was titrated into the sample cell containing thiol **3** or **4** (0.2 mM). Thermodynamic parameters shown based on triplicate binding assays and the reported as mean ± SE.

3. Conclusion

While small molecule thio-carbonyl compounds have previously been shown to inhibit various MBLs, their ability to synergize with β-lactam antibiotics in overcoming MBL-associated resistance has not been extensively studied. We here demonstrate a significant level of synergism between meropenem and a series of thiols, most notably thiomandelic acid **3** and 2-merpto-3-phenylpropionic acid **4**. Combinations of meropenem with **3** or **4** exhibit antibacterial activity against a number of gram-negative bacteria expressing different MBLs including IMP, NDM and VIM. Given the high degree of active site heterogeneity among the different types of MBL enzymes,[11] designing an inhibitor with potent inhibitory activity towards several types of MBL is challenging. In this light, thiomandelic acid **3** is unique given its ability to inhibit a range of MBLs and, as shown in the present study, its capacity to resensitize MBL-expressing gram-negative isolates to meropenem, an important β-lactam antibiotic of last resort. In addition, ITC studies showed thiols **3** and **4** to be effective zinc chelators with low-micromolar K_d values supporting the proposed mechanism of action for these compounds. In this regard, ITC may provide a useful means of (pre)screening for zinc-binding MBL inhibitors. While compounds **3** and **4** exhibit potent synergy with meropenem, their propensity to oxidize and likely ability to interact with free zinc and other metallo-proteins precludes their use as clinical MBL inhibitors. In this regard, MBL inhibitors employing free thiols as zinc binding groups are more likely to be of value as tool compounds for biochemical studies involving MBLs. Optimized analogs or other classes of MBL inhibitors capable of overcoming such pharmacokinetic hurdles present a key objective in the continued fight against antibiotic resistance.

4. Experimental Section

General

Potassium thioacetate was purchased from Combi-Blocks Inc. (San Diego, CA USA). Thiosalicylic acid was purchased from Fisher Scientific (Waltham, MA USA). Other reagents including captopril, 2-bromoacetophenone, *S*-mandelic acid, *L*-phenylalanine, were purchased from Sigma-Aldrich company. The reaction progress was monitored by thin-layer chromatography (normal SiO_2, Merck 60 F254) and ethyl acetate/petroleum ether combination was used as developing phase. The plates were visualized using UV indicator and/or stained by ceric ammonium molybdate reagent. The NMR spectra were obtained on an Agilent 400 MHz spectrometer. DMSO(d_6) or $CDCl_3$ were used to dissolve the samples and tetramethylsilane was used as internal standard. High-resolution mass spectrometry was performed using an ESI instrument. As the only exception, the molecular ion of thiol **5** could not be detected by the ESI technique.

Synthesis

Among the thiols selected for investigation, captopril **1** and thiosalicylic acid **2** were commercially available while compounds **3**-**5** required preparation via previously reported synthetic routes.[21,30–32] Briefly, compound **3** was synthesized via esterification of *S*-mandelic acid which was followed by mesylation of the hydroxyl group. Substitution of the tosylate ester with thioacetate anion followed by acidic hydrolysis furnished **3**.[30] For the synthesis of compound **4**, *L*-phenylalanine was converted to its corresponding α-bromocarboxylic acid through a sodium nitrite mediated halo-deamination reaction, which was subsequently reacted with potassium thioacetate to afford S-acetyl derivative of **4**. Basic hydrolysis of the latter intermediate led to the final product **4**.[31,32] Thioacetophenone **5** was prepared via a two-step procedure involving thiolation of α-bromoacetophenone with potassium thioacetate followed by a basic hydrolysis.[21] In addition, disulfides **6**-**8**[33–35] were readily prepared by reacting the corresponding thiol with iodine in water/acetonitrile.[36]

Spectral characterization of compounds 3-8.

3	^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.47–7.42 (m, phenyl H, 2H), 7.38–7.29 (m, phenyl H, 3H), 4.69 (d, J = 7.6 Hz, -CH-, 1H), 2.60 (d, J = 7.6 Hz, -SH, 1H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 177.62, 137.13, 128.86, 128.49, 127.86, 45.45. HRMS (ESI): $[M-H]^-$ calculated: 167.0178, found: 167.0172.
4	^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.39–7.11 (m, phenyl H, 5H), 3.62 (m, aliphatic H, 1H), 3.25 (dd, J = 14.0 Hz, J = 8.2 Hz, aliphatic H, 1H), 3.01 (dd, J = 14.0 Hz, J = 7.0 Hz, aliphatic H, 1H), 2.15 (d, J = 8.9 Hz, -SH, 1H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 179.03, 137.26, 129.06, 128.64, 127.15, 42.22, 41.11. HRMS (ESI): $[M-H]^-$ calculated: 181.0329, found: 181.0318.
5	^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.95 (m, phenyl H, 2H), 7.58 (m, phenyl H, 1H), 7.47 (t, J = 8.0 Hz, phenyl H, 2H), 3.95 (d, J = 7.3 Hz, -CH_2-, 2H), 2.12 (t, J = 7.3 Hz, -SH, 1H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 194.71, 135.01, 133.59, 128.79, 128.47, 31.11.
6	^{1}H-NMR (400 MHz, Acetone-d_6): Isomeric mixture (7:1) δ 7.46–7.35 (m, phenyl H, 10H), 4.79 and 4.71 (each s, -CH, isomeric ratio 1:7 respectively). ^{13}C-NMR (100 MHz, DMSO-d_6): δ 171.40, 171.34, 136.10, 136.05, 129.12, 129.10, 129.01, 128.84, 128.79, 57.77, 57.59. HRMS (ESI): $[M-H]^-$ calculated: 333.0261, found: 333.0259.
7	^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.29-7.14 (m, 10H, phenyl H), 3.65 (t, J = 7.7 Hz, aliphatic H, 2H), 3.22 (dd, J = 14.3 Hz, J = 8.2 Hz, aliphatic H, 2H), 3.01 (dd, J = 14.3 Hz, J = 7.2 Hz, aliphatic H, 2H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 177.57, 136.70, 129.03, 128.62, 127.07, 53.03, 36.75. HRMS (ESI): $[M-H]^-$ calculated: 361.0574, found: 361.0569.
8	^{1}H-NMR (400 MHz, $CDCl_3$): δ 7.92 (m, phenyl H, 4H), 7.56 (m, phenyl H, 2H), 7.45 (m, phenyl H, 4H), 4.18 (s, CH_2, 4H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 194.27, 135.35, 133.64, 128.73, 128.69, 45.38. HRMS (ESI): $[M+Na]^+$ calculated: 325.0333, found: 325.0338.

MIC determinations and synergy assays

The antibacterial activity of compounds **1**-**5** was evaluated alone and in combination with meropenem against a panel of β-lactamase producing gram-negative bacteria including *K. pneumoniae* RC10 (KPC-2), *K. pneumoniae* RC 45 (OXA-48), *K. pneumoniae* RC51 (VIM-1), *K. pneumoniae* JS265 (IMP-28), *E. coli* RC89 (NDM-1), *P. aeruginosa* RC60 (VIM-2), *P. aeruginosa* JS80 (IMP13, IMP-37), *K. pneumoniae* RC21 (VIM-1), *E. aerogenes* RC22 (VIM-1), *K. pneumoniae* RC48 (VIM-1), *K. pneumoniae* JS22 (NDM-1), *K. pneumoniae* JS177 (NDM-1), and *K. pneumoniae* JS37 (NDM-1). The CLSI guidelines were used to determine minimum inhibitory concentrations (MICs). Starting from glycerol stocks, bacterial strains were cultured on blood agar plates and incubated at 37 °C. A single colony was then transferred to tryptic soy broth (TSB) and incubated with shaking at 37 °C until the optical density of the bacterial suspension reached a level equivalent to the 0.5 McFarland standard. The suspension was then diluted to 10^6 CFU/mL in Mueller-Hinton broth (MHB). Using polypropylene microtiter plates, the wells of the first row received 50 μL of the test compounds dissolved in MHB and were subjected to serial dilution. Finally, 50 μL of the bacterial suspension was added and the plates were sealed and incubated at 37 °C with constant shaking (at 600 RPM). The next morning, the plates were inspected for visible bacterial growth (see table 3 for antibiotic susceptibility data).

Synergy between meropenem (or cefoperazone) and thiols **1**-**5** was evaluated as follows: To the top row of a 96-well plate, 100 μL of a solution of meropenem at 4×MIC was added. 50 μL aliquots of this solution was serially diluted down each row to achieve a range of decreasing meropenem concentrations. Next, thiols **1**-**5** (50 μL aliquots) were added to the wells to provide a concentration range of 128 to 16 μg/mL. The relevant bacterial suspension (100 μL/well) was finally added to each well to give a final concentration of thiols **1**-**5** ranging from 64 to 8 μg/mL. The plates were sealed and incubated overnight at 37 °C. MICs were determined the next morning by visual inspection and used in calculating fractional inhibitory concentration index (FICI) values. As a standard MBL inhibitor cocktail, a 16:1 (w/w) mixture of ethylenediaminetetraacetic acid disodium salt-phenanthroline (EP) was also used. The MIC of the EP mixture was first determined against each strain so that a sub-MIC concentrations could be used for synergy assays. All the assays were performed in duplicates. Figure 4 provides a graphical representation of the checkerboard assays.

Table 3. Antibiotic susceptibility of the MBL-producing isolates

Isolates	MIC (μg/mL)					
	Meropenem	Cefoperazone	Ciprofloxacin	Gentamicin	Tobramycin	Colistin
K. pneumoniae (KPC-2)	>128 (R)[a]	>256 (R)	≥4 (R)[b]	4 (S)[b]	≥16 (R)[b]	1 (S)[b]
K. pneumoniae (OXA-48)	64 (R)	>256 (R)	>32 (R)	>32 (R)	32 (R)	≤0.25 (S)
K. pneumoniae (VIM-1)	64/32 (R)	>256 (R)	>32 (R)	>32 (R)	32 (R)	32 (R)[b]
K. pneumoniae (IMP-28)	16/8 (R)	256 (R)	1 (R)	0.5 (S)	1 (S)	≤0.25 (S)
E. coli (NDM-1)	128/64 (R)	>256 (R)	>32 (R)	>32 (R)	>32 (R)	≤0.25 (S)
P. aeruginosa (VIM-2)	32 (R)	128 (R)	≥4 (R)[b]	≥16 (R)[b]	≥16 (R)[b]	1 (S)[b]
P. aeruginosa (IMP-13, IMP-37)	64 (R)	256 (R)	32 (R)	>32 (R)	32 (R)	1 (S)
K. pneumoniae (VIM-1)	64/32 (R)	>256 (R)	≥4 (R)[b]	≥16 (R)[b]	≥16 (R)[b]	≤0.5 (S)[b]
E. aerogenes (VIM-1)	32/16 (R)	>256 (R)	1 (R)[b]	≤1 (S)[b]	≥16 (R)[b]	≤0.05 (S)[b]
K. pneumoniae (VIM-1)	>128/128 (R)	>256 (R)	≥4 (R)[b]	2 (S)[b]	16 (R)	≤0.5 (S)[b]
K. pneumoniae (NDM-1)	32/16 (R)	>256 (R)	≥8 (R)[b]	≥16 (R)[b]	≥4 (R)[b]	≤1 (S)[b]
K. pneumoniae (NDM-1)	16/8 (R)	>256 (R)	>32 (R)	>32 (R)	16 (R)	8 (R)
K. pneumoniae (NDM-1)	64/32 (R)	>256 (R)	≥8 (R)[b]	≥16 (R)[b]	≥4 (R)[b]	≤1 (S)[b]

[a]R: resistant; S: sensitive. Sensitivity to the tested antibiotics (except for cefoperazone) according to the clinical MIC breakpoints issued by the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[37] For cefoperazone, the MIC breakpoint based on that published by the United States food and drug administration (FDA).[38]

[b]MIC data provided by Utrecht University medical center.

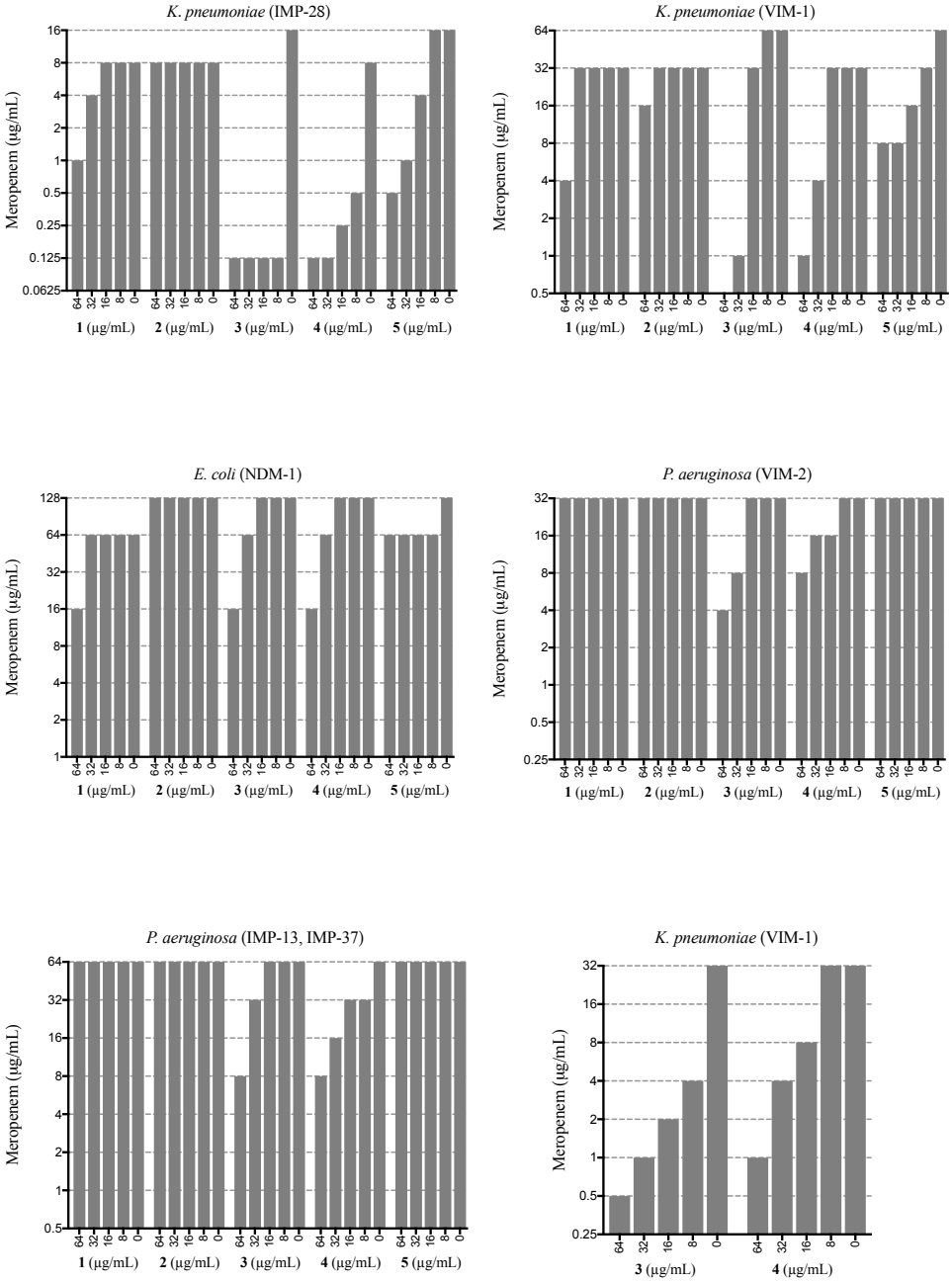


Figure 4. The MIC values of meropenem/cefoperazone in combination with thiols **1**-**5**.

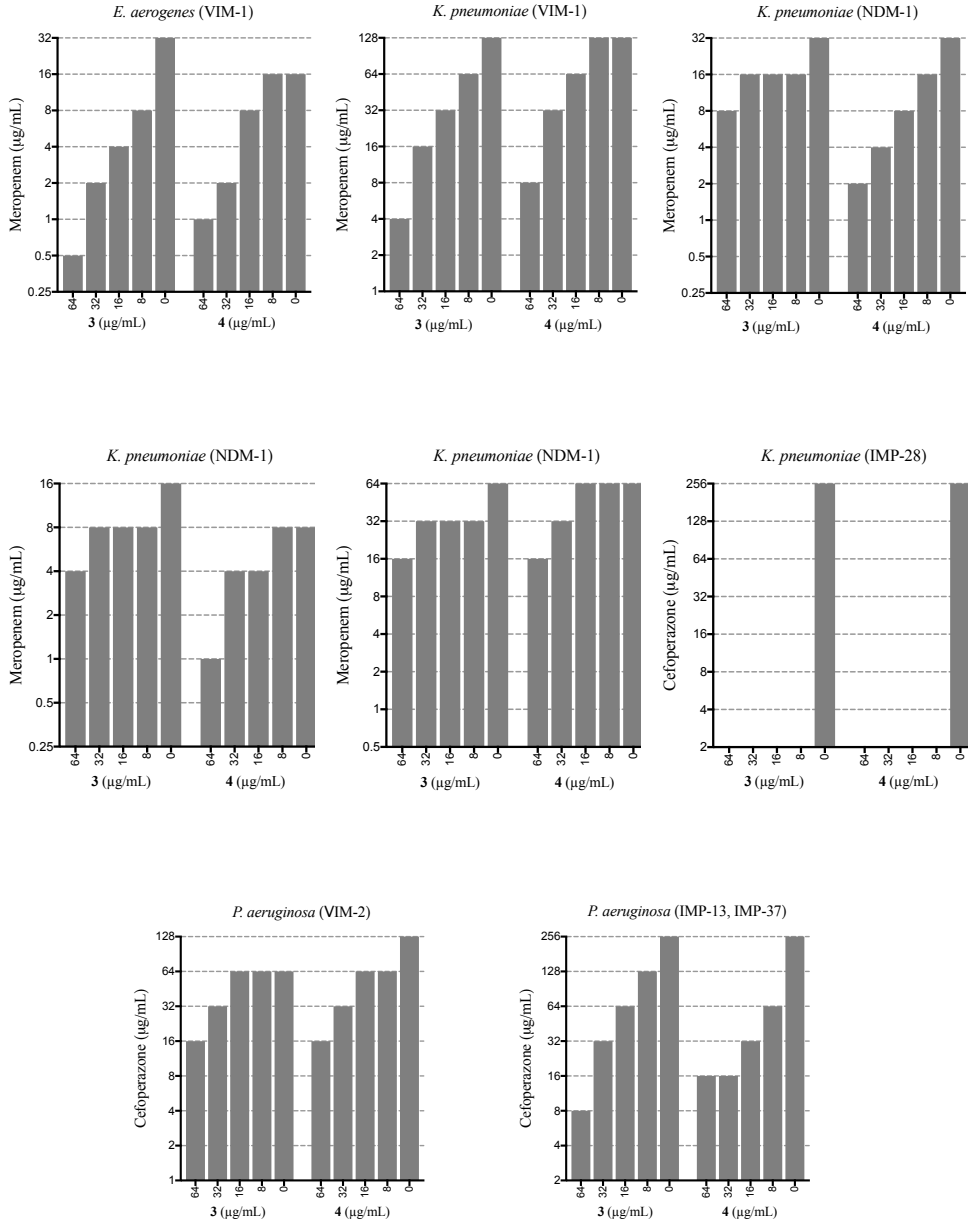


Figure 4. *Continued.*

Stability analysis

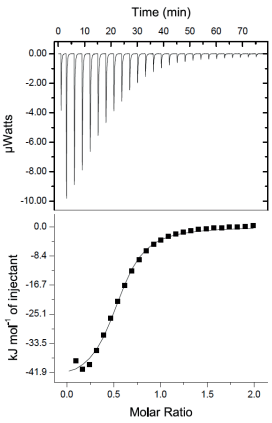
To prepare calibration curves for thiols **3**-**5** and the corresponding disulfides **6**-**8** each compound was dissolved in DMSO to prepare a stock solution (10 mg/mL) that was then immediately diluted in Mueller-Hinton broth (MHB) to reach concentrations ranging from 1 μg/mL to 256 μg/mL. The samples were then processed as follows: To precipitate undesired media components, the sample solutions were diluted with acetonitrile (1:3 v/v), vortexed for 10 seconds, and centrifuged at 10000 rpm for 10 min. The supernatant was retained and stored at -20 °C until HPLC analysis. Samples were analyzed by analytical RP-HPLC using a Phenomenex Gemini C-18 110A column (250×4.60 mm, 5 micron) at a flow rate of 1 mL/min and their UV absorbance were detected at 214 nm. For the analysis of compounds **4**, **5**, **7**, and **8**, the gradient started with 0% of buffer B (5% H_2O, 95% acetonitrile, 0.1% TFA) and 100% buffer A (95% H_2O, 5% acetonitrile, 0.1% TFA) increasing to 50% buffer B over 5 min followed by an increase to 100% buffer B over 10 min and maintenance at 100% buffer B for 3 min. The buffer gradient was then returned to 0% buffer B in 2 min and maintained at 0% for an additional 5 min to re-equilibriate the system. For the analysis of compounds **3** and **6** the gradient started with 0% of buffer B, increased to 50% buffer B over 5 min, then to 90% buffer B over 8 min followed by a final increase to 100% buffer B over 1 min. After 1 min at 100% buffer B the gradient returned to 0% buffer B over 2 min and was maintained at 0% for 3 min to re-equilibriate the system. The calibration curves were linear from 2-256 μg/mL for compounds **3**-**6** and from 1.6-200 μg/mL for compounds **7** and **8** with $r^2 > 0.990$ in all the cases. Due to the relatively short half-life of compound **5** in MHB, the medium needed to be supplemented with 5.0 mM TCEP to obtain a suitable calibration curve. To assess the half-lives of thiols **3**-**5** in MHB, each compound was dissolved in MHB (200 μg/mL) and incubated at 37 °C. At time points of 0, 0.5, 1, 2, 4 and 8 hours, 100 μL aliquots of each samples were taken and subjected to the same processing described above for the standards prior to HPLC analysis.

Isothermal titration calorimetry

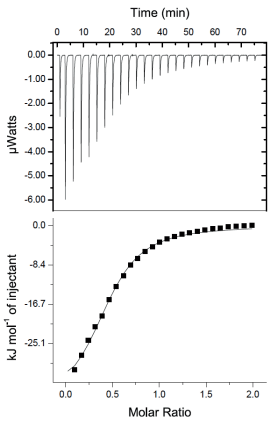
ITC experiments were performed using a MicroCal Auto-ITC200 instrument (Malvern). The test compounds and zinc chloride were dissolved in Tris-HCl buffer (20 mM, pH 7.0) and degassed using a sonication bath (10 min) before running the experiments. The zinc chloride solution was titrated into a solution of **3**, **4**, **6**, **7**, EDTA, or 1,10-phenanthroline (see table 4 for specific concentrations used) over 26 aliquots of 1.5 μL (except the first injection which was 0.5 μL) with 120 seconds spacing between injections. All the experiments were performed at 25 °C in triplicate with reference power set at 2.0 μcal/sec. The generated peaks were integrated using Origin 7.0 software (see figure 5 for thermograms). The error for all the reported thermodynamic parameters was estimated through Monte Carlo simulation the standard errors of three experiments.

Table 4. Concentrations of the metal/ligands used for the ITC experiments

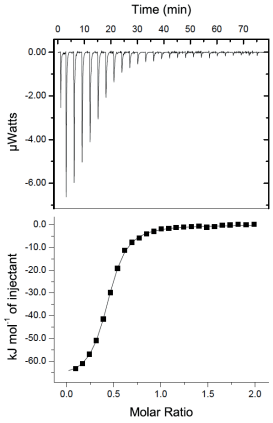
Experiment (metal/ligand)	Zn^{2+} concentration (mM)	Ligand concentration (mM)
$ZnCl_2$ / compound **3**,**4**,**6**,**7**	2.0	2.0
$ZnCl_2$ / EDTA	0.4	0.04
$ZnCl_2$ / phenanthroline	1.0	0.1



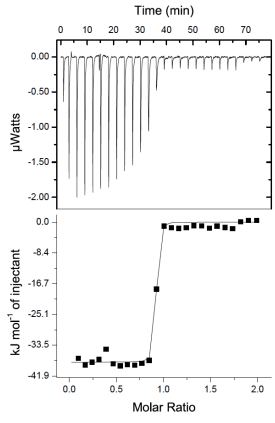
$ZnCl_2$ (2.0 mM) : **3** (0.2 mM)



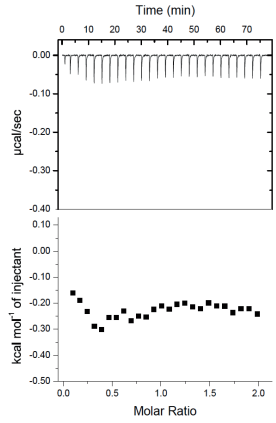
$ZnCl_2$ (2.0 mM) : **4** (0.2 mM)



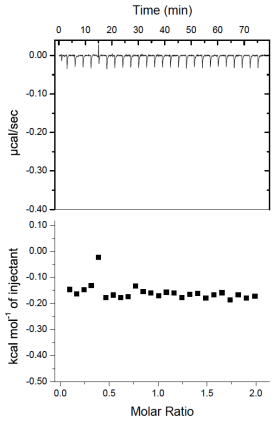
$ZnCl_2$ (1.0 mM) : 1,10-phenanthroline (0.1 mM)



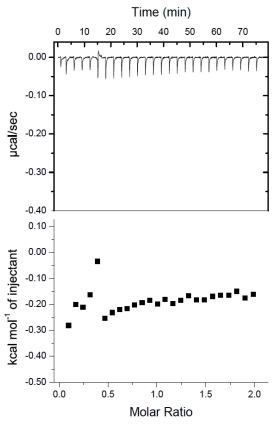
$ZnCl_2$ (0.4 mM) : EDTA (0.04 mM)



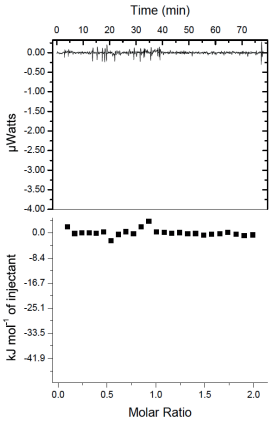
Buffer : **3** (0.2 mM)



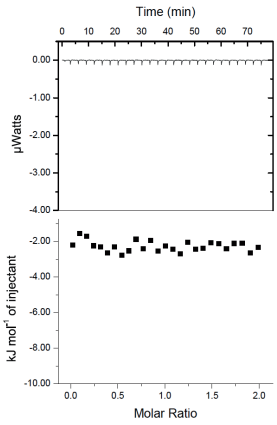
Buffer : **4** (0.2 mM)



$ZnCl_2$ (2.0 mM) : Buffer



Buffer : 1,10-phenanthroline (0.1 mM)



Buffer : EDTA (0.04 mM)

Figure 5. The ITC thermograms

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