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# Mechanistic Investigations of Metallo- $\beta$ -lactamase Inhibitors: Strong Zinc Binding Is Not Required for Potent Enzyme Inhibition\*\*

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Metallo- $\beta$ -lactamases (MBLs) are zinc-dependent bacterial enzymes that inactivate essentially all classes of  $\beta$ -lactam antibiotics including last-resort carbapenems. At present there are no clinically approved MBL inhibitors, and in order to develop such agents it is essential to understand their inhibitory mechanisms. Herein, we describe a comprehensive mechanistic study of a panel of structurally distinct MBL inhibitors reported in both the scientific and patent literature. Specifically, we determined the half-maximal inhibitory concentration ( $IC_{50}$ ) for each inhibitor against MBLs belonging to the NDM and IMP families. In addition, the binding affinities of the inhibitors for

$Zn^{2+}$ ,  $Ca^{2+}$  and  $Mg^{2+}$  were assessed by using isothermal titration calorimetry (ITC). We also compared the ability of the different inhibitors to resensitize a highly resistant MBL-expressing *Escherichia coli* strain to meropenem. These investigations reveal clear differences between the MBL inhibitors studied in terms of their  $IC_{50}$  value, metal binding ability, and capacity to synergize with meropenem. Notably, our studies demonstrate that potent MBL inhibition and synergy with meropenem are not explicitly dependent on the capacity of an inhibitor to strongly chelate zinc.

## Introduction

Antibiotics revolutionized health care in the mid-20th century and they continue to be a cornerstone of modern medicine. Among all classes of antibiotics, the  $\beta$ -lactams are the most widely used, with broad-spectrum penicillins and cephalosporins accounting for 55% of antibiotics consumed in 2010.<sup>[1]</sup> Unfortunately, the widespread use of  $\beta$ -lactams over the past decades has led to the emergence of bacterial pathogens with resistance to all classes of  $\beta$ -lactam antibiotics used today.<sup>[2]</sup> The most notable mode of  $\beta$ -lactam resistance is via the production of  $\beta$ -lactamase enzymes that catalyze the opening of the  $\beta$ -lactam ring. The  $\beta$ -lactamases can be mechanistically divided into two types; serine  $\beta$ -lactamases (SBLs, Ambler class A, C and D) and metallo- $\beta$ -lactamases (MBLs, Ambler class B).<sup>[3]</sup> Inhibitors of the SBLs have been successfully developed and include the

clinically used clavulanic acid, sulbactam, tazobactam, and the most recent avibactam and vaborbactam.<sup>[4]</sup> MBLs, however, are mechanistically different from SBLs and rely on active site  $Zn^{2+}$  ion(s) to activate a water molecule that in turn hydrolyzes the  $\beta$ -lactam ring.<sup>[5]</sup> This mechanistic difference means that clinically used SBL inhibitors have little-to-no effect on MBLs. Currently there are no clinically approved MBL inhibitors.

Among the known MBLs the so-called New Delhi metallo- $\beta$ -lactamase (NDM) and imipenemase (IMP) enzymes are among the best studied and confer resistance to a wide range of  $\beta$ -lactam antibiotics. The active site of both the NDM and IMP families are conserved and contain one  $Zn^{2+}$  ion coordinated to three histidine side chains and a second  $Zn^{2+}$  coordinated by histidine, aspartic acid, and cysteine residues.<sup>[6,7]</sup> While NDM and IMP enzymes share structural similarities, their primary amino acid sequences differ significantly leading to differences in both their catalytic efficiency and sensitivity to small molecule inhibitors.<sup>[8–11]</sup>

The present lack of any clinically approved MBL inhibitor emphasizes the need for investigation and innovation in this area. Ideally, an MBL inhibitor would be administered as part of a combination therapy with the capacity to restore the activity of a  $\beta$ -lactam antibiotic against otherwise resistant bacteria expressing an MBL. While no MBL inhibitor has yet been granted approval for use in humans, a wide range of inhibitors, spanning a diversity of structural and mechanistic features, have been reported in both the scientific and patent literature in recent years. The structures of these reported MBL inhibitors contain a wide variety of pharmacophores that can deactivate the MBLs most often by either zinc-sequestration (i.e., “zinc stripping”) or by coordination to zinc as part of the compound’s interaction within the MBL active site.<sup>[12]</sup> Strong metal chelators

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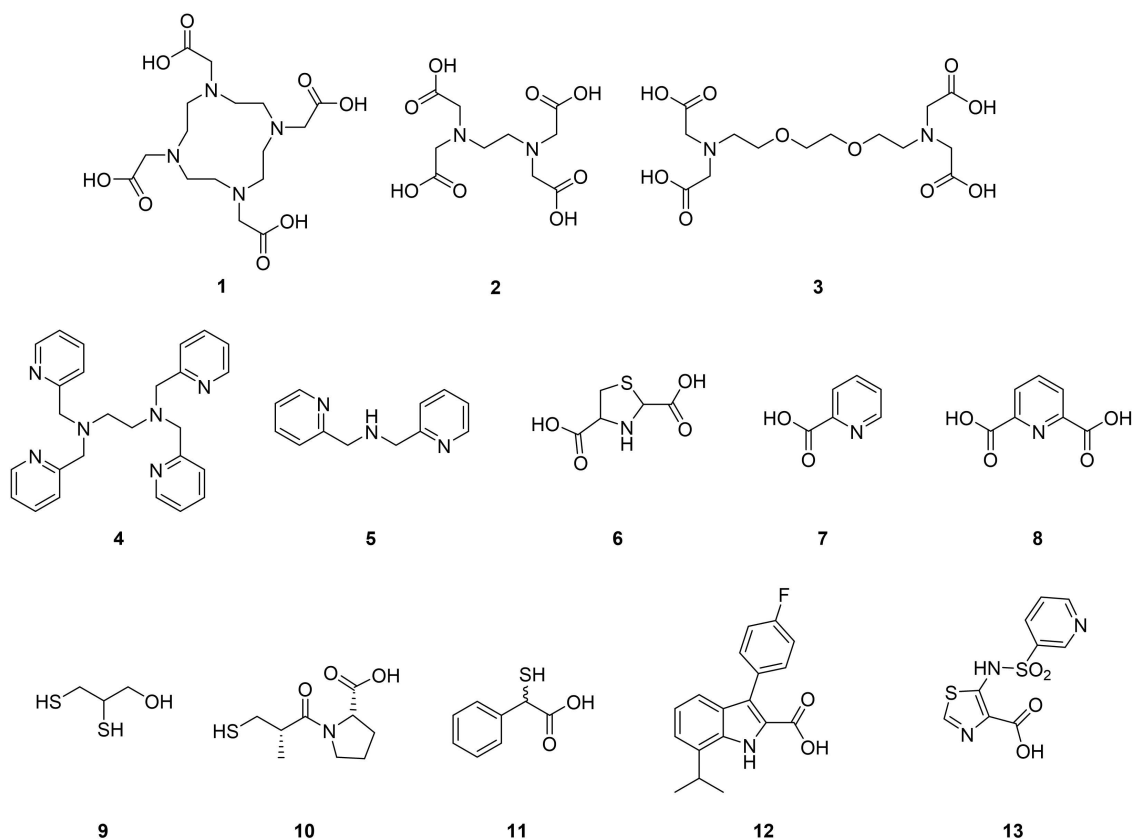
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that actively strip zinc ions from the MBL active site can effectively deactivate the enzyme's ability to hydrolyze the  $\beta$ -lactam ring.<sup>[13]</sup> Alternatively, there are also small molecule MBL inhibitors that instead of removing zinc from the enzyme, coordinate the metal ion within the active site and in doing so displace the activated water molecule which in turn blocks catalysis.<sup>[7,14]</sup>

Developing inhibitors that effectively cover all MBL subtypes is a challenge with many factors to consider. As the MBLs are metallo-enzymes, searching for inhibitors with the capacity to chelate or bind the active site zinc ions would appear to be an obvious strategy. However, there is a significant risk involved with the use of zinc binding compounds *in vivo* given the possibility of off-target effects related to the many other metallo-enzymes involved in human metabolism.<sup>[15,16]</sup> Among the earliest reported MBL inhibitors were various thiol and thio-carbonyl compounds which demonstrated potent *in vitro* enzyme inhibition for a range of MBLs.<sup>[17]</sup> However, the rapid oxidation of thiols to homo- and hetero-disulfides in biological systems can negatively affect their clinical success.<sup>[18,19]</sup> Ideally, an MBL inhibitor should be stable enough for use *in vivo* and also not interfere with the binding of biologically relevant metals needed for the function of other vital metallo-enzymes.

Given the above-mentioned challenges associated with the discovery of MBL inhibitors, it is crucial to understand the physicochemical (specifically metal-binding) properties of MBL

inhibitors in order to clarify their inhibitory mechanism, as well as their likelihood of having off-target effects if administered *in vivo*. To this end, we surveyed the recent scientific and patent literature and assembled a broad panel of 13 structurally distinct MBL inhibitors for the purpose of a comparative study. Specifically, we set out to: 1) Establish the relative inhibitory potency of these MBL inhibitors in a directly comparative manner (i.e., using the same biochemical assay) by determining the  $IC_{50}$  values for each inhibitor against purified NDM-1 and IMP-1; 2) Assess the metal binding properties of the MBL inhibitors using isothermal titration calorimetry (ITC) to quantify their affinity for the biologically relevant divalent cations  $Zn^{2+}$ ,  $Ca^{2+}$ , and  $Mg^{2+}$ ; and 3) Assess the ability of these MBL inhibitors to synergize with a last resort carbapenem antibiotic (meropenem) in resensitizing an *Escherichia coli* strain expressing NDM-1. Figure 1 provides an overview of the structures of the MBL inhibitors selected for the present study. The strong metal chelators, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) **1**, ethylenediaminetetraacetic acid (EDTA) **2** and ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) **3** are all known to have a high affinity for divalent cations making them ideal candidates to study as inhibitors that inactivate MBLs by zinc stripping.<sup>[10,11,20]</sup> The pyridine based *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) **4** and di-(2-picoly)amine **5** were also included as they have been described as MBL inhibitors with the ability to bind zinc.<sup>[21]</sup>



**Figure 1.** Structures of inhibitors: heavy metal chelators (1–4), pyridine-based chelators (4 and 5), *N*-heterocyclic carboxylic acids (6–8), thiols (9–11) and HTS derived inhibitors (12 and 13).

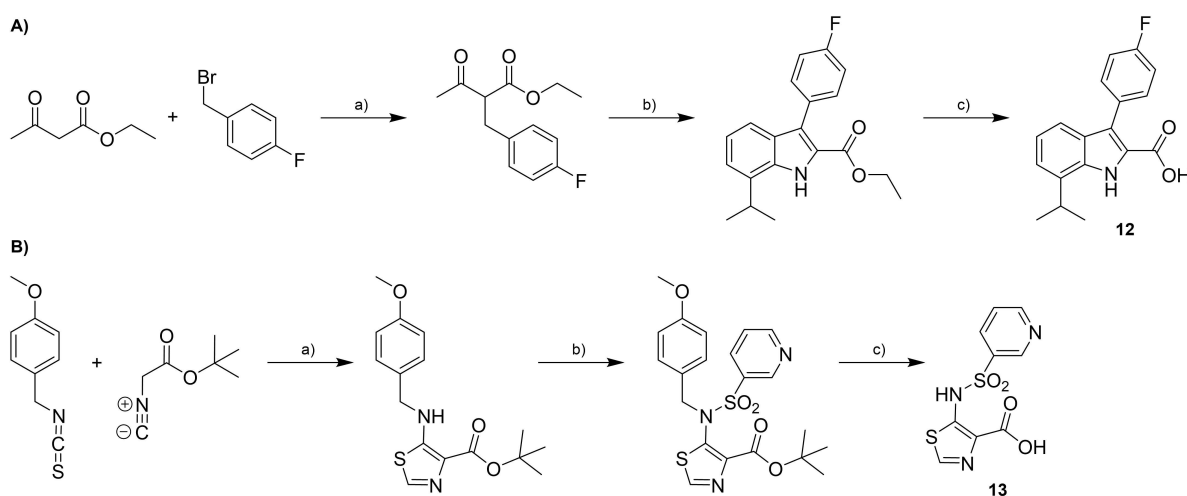
Another category of compounds included were the *N*-heterocycle carboxylates thiazolidine-2,4-dicarboxylic acid **6**, picolinic acid **7**, and dipicolinic acid (DPA) **8** which have been shown to inhibit MBL enzymes such as NDM-1 and CphA.<sup>[22,23]</sup> As representative members of the thiol-based MBL inhibitors, dimercaprol **9**, L-captopril **10**, and thiomandelic acid **11** were selected.<sup>[14,22,24–27]</sup> In addition, compounds **12** and **13** were included as both were recently reported in the patent literature as displaying potent MBL inhibition.<sup>[28,29]</sup> Distinct from the other MBL inhibitors chosen for this study, compounds **12** and **13** are the result of high-throughput screening and medicinal chemistry efforts specifically aimed at identifying MBL inhibitors. Compound **12** comes from a library of inhibitors all containing an indole-2-carboxylate core, shown to have potent NDM-1 inhibition.<sup>[28]</sup> Compound **13** demonstrates promising *in vitro* inhibition of NDM-1 and Verona integron-encoded metallo- $\beta$ -lactamase 2 (VIM-2) and is under development by the biopharmaceutical company Antabio. A recent co-crystal structure of compound **13** in complex with VIM-2 shows that the compound interacts with the MBL active site via  $Zn^{2+}$  coordination by the carboxylate moiety and thiazole nitrogen atom in addition to interacting with active site residues.<sup>[30]</sup>

In light of the growing interest in MBL inhibitor development we here provide a comparative analysis of the biochemical, biophysical, and biological properties of a representative set of MBL inhibitors. Notably, our study provides for a direct assessment of the relative activity of these inhibitors against the NDM and IMP type enzymes. A number of studies published in recent years have used a variety of assay conditions to establish MBL inhibitor potency and synergy. In contrast, our investigation employs the exact same assay conditions in assessing the inhibitory activities of the MBL inhibitors studied and in doing so provides for a reliable comparison. In addition, in determining the metal binding affinities of the MBL inhibitors here studied, we reveal the somewhat surprisingly finding that tight zinc binding is by no means a prerequisite for potent enzyme

inhibition. Notably, at the time of writing a manuscript appeared from Crowder and co-workers describing the use of equilibrium dialyses with metal analyses, native state electrospray ionization mass spectrometry (ESI-MS), and UV-Vis spectrophotometry to compare the activity and mechanism of various MBL inhibitors towards VIM-2.<sup>[31]</sup> The techniques used and results reported in the Crowder group's study with VIM-2 are complimentary to the methods we use and support well the findings here described in our comparative analysis of MBL inhibitors towards the NDM and IMP classes.

## Results and Discussion

Among the MBL inhibitors used in the present study, compounds **1–11** are commercially available, while **12** and **13** are not and were therefore synthesized. Compound **12** was prepared according to patent literature by the route shown in Scheme 1A.<sup>[28]</sup> Briefly, ethyl acetoacetate was alkylated with 4-fluorobenzyl bromide to yield the expected substituted  $\beta$ -ketoester. The next step involved the *in situ* diazotization of 2-isopropylaniline, which, in the presence of the substituted  $\beta$ -ketoester intermediate from the previous step, resulted in hydrazone formation (Japp-Klingemann reaction) followed by spontaneous cyclization under acidic conditions to form the indole scaffold. Ester hydrolysis in turn gave compound **12** (experimental details provided in the Supporting Information). The synthesis of compound **13** was achieved following a modified protocol from the patent literature and is illustrated in Scheme 1B.<sup>[29]</sup> The route started by cyclization of 4-methoxybenzyl isothiocyanate with *tert*-butyl isocynoacetate to yield the thiazole core. Subsequent reaction of this intermediate with pyridine-3-sulfonyl chloride afforded the corresponding sulfonamide. Notably, yields for the formation of the sulfonamide were best when starting from the PMB protected thiazole intermediate and using potassium bis(trimethylsilyl)-amine as a



**Scheme 1.** A) Synthesis of compound **12**. a) ethyl acetoacetate, 4-fluorobenzylbromide, potassium *tert*-butoxide, *tert*-butanol, THF; b) *i*. 2-isopropylaniline, sodium nitrate, conc. HCl, MeCN; ii. KOH, H<sub>2</sub>O, EtOH; iii. HCl, EtOH; c) NaOH, THF, EtOH. B) Synthesis of compound **13**. a) potassium *tert*-butoxide, THF; b) pyridine-3-sulfonyl chloride, potassium bis(trimethylsilyl)amide, DMF/THF; c) TFA.

base. Following sulfonamide formation, global deprotection using acidic conditions led to compound **13** (experimental details are provided in the Supporting Information).

With inhibitors **1–13** in hand we next determined their half-maximal inhibitory concentrations ( $IC_{50}$  values) against purified NDM-1 and IMP-1 enzymes. For these assays, the fluorogenic cephalosporin substrate FC5 was synthesized and used as previously described.<sup>[32]</sup> The  $K_M$  and  $k_{cat}$  values for FC5 hydrolysis by NDM-1 corresponded with those previously found.<sup>[33]</sup> The results of this study, shown in Table 1, highlight differences between the compounds studied and their ability to inhibit the two enzymes. The metal chelating compounds **1–5** are particularly strong inhibitors of NDM-1 but are significantly less effective against IMP-1. The heterocyclic carboxylates **6–8** also showed NDM-1 selectivity among which DPA **8** was the most potent against both enzymes. Thiols **9–11** were found to have low- to sub- $\mu$ M  $IC_{50}$  values against both NDM-1 and IMP-1. Among these, thiomandelic acid **11** was the most effective with  $IC_{50}$  values of 3.2 and 0.02  $\mu$ M against NDM-1 and IMP-1, respectively. Among the MBL inhibitors selected from the

recent patent literature, indole-carboxylate **12** showed an impressive potency activity against both NDM-1 and IMP-1 with  $IC_{50}$  values in the nanomolar range. Notably, while inhibitor **13** exhibited nanomolar potency against NDM-1 it was found to be significantly less active towards IMP-1.

The ability of inhibitors **1–13** to bind the biologically relevant divalent cations  $Zn^{2+}$ ,  $Ca^{2+}$ , and  $Mg^{2+}$  was next investigated. Understanding the metal binding properties of MBL inhibitors is of importance when considering their potential for therapeutic application given the high concentrations of free calcium and magnesium in the bloodstream, with values in adult humans of between 1.17–1.33 and 0.6–1.1 mM, respectively.<sup>[34,35]</sup> These ions have the potential to “distract” metal binding inhibitors from reaching the divalent zinc in the MBL targets. A range of methodologies and conditions have been used previously in evaluating the metal-binding properties of some of the inhibitors included in our present study.<sup>[18,36–41]</sup> In an attempt to employ a more standardized approach, we used isothermal titration calorimetry to determine and compare the thermodynamic parameters governing the binding interaction between inhibitors **1–13** and the divalent cations  $Zn^{2+}$ ,  $Ca^{2+}$ , and  $Mg^{2+}$ . Listed in Table 2 are the values resulting from our ITC binding studies including: dissociation constant ( $K_d$ ), enthalpy ( $\Delta H$ ), entropy ( $-T\Delta S$ ), and Gibbs free energy ( $\Delta G$ ); all binding thermograms are provided in the Supporting Information, see also Figure S2 in the Supporting Information). In all cases, the MBL inhibitor evaluated showed much more potent zinc binding relative to either calcium or magnesium. Notably, the Zn-binding interaction of compounds **1–4** is very strong (estimated  $K_d < 100$  nM) precluding an accurate determination of their zinc binding constants based on the data obtained by ITC. The strong metal ion binding observed for **1–4** is in fact expected given the well-established capacity of these compounds to act as potent chelating agents, all with  $K_d < 1$  nM as previously determined.<sup>[36,37]</sup> Also of note are compounds **5** and **9**, which exhibit an apparent two-step binding interaction with zinc.

**Table 1.**  $IC_{50}$  values of the inhibitor against either NDM-1 or IMP-1.

Compound	$IC_{50}$ [a] [ $\mu$ M]	
	NDM-1	IMP-1
<b>1</b>	1.34 $\pm$ 0.06	> 200
<b>2</b>	1.97 $\pm$ 0.06	> 200
<b>3</b>	0.280 $\pm$ 0.016	> 200
<b>4</b>	0.644 $\pm$ 0.245	109 $\pm$ 11.3
<b>5</b>	2.60 $\pm$ 0.17	116 $\pm$ 0.9
<b>6</b>	55.0 $\pm$ 5.92	> 200
<b>7</b>	84.0 $\pm$ 7.25	> 200
<b>8</b>	4.26 $\pm$ 0.28	15.2 $\pm$ 0.168
<b>9</b>	4.21 $\pm$ 0.40	2.19 $\pm$ 0.070
<b>10</b>	7.21 $\pm$ 0.88	3.48 $\pm$ 0.254
<b>11</b>	3.17 $\pm$ 0.06	0.023 $\pm$ 0.001
<b>12</b>	0.005 $\pm$ 0.002	0.140 $\pm$ 0.003
<b>13</b>	0.275 $\pm$ 0.029	> 200

[a] The half-maximal inhibitory concentration values for each compound against NDM-1 and IMP-1 with FC5 used as the substrate. Values shown are averages of triplicate measurements  $\pm$  SD.

**Table 2.** Binding of  $Zn^{2+}$  by MBL inhibitors **1–13** as assessed using isothermal titration calorimetry.

Compound	$K_d$ [ $\mu$ M]	$\Delta H$ [kcal/mol]	$-T\Delta S$ [kcal/mol]	$\Delta G$ [kcal/mol]
<b>1</b>	< 100 nM <sup>[a]</sup>	-11.3 $\pm$ 0.09	-	-
<b>2</b>	< 100 nM <sup>[a]</sup>	-8.80 $\pm$ 0.10	-	-
<b>3</b>	< 100 nM <sup>[a]</sup>	-12.3 $\pm$ 0.06	-	-
<b>4</b>	< 100 nM <sup>[a]</sup>	-12.0 $\pm$ 0.07	-	-
<b>5</b>	0.139 $\pm$ 0.062	-13 $\pm$ 0.08	3.61 $\pm$ 0.18	-9.41 $\pm$ 0.25
	1.47 $\pm$ 0.72	-2.49 $\pm$ 0.26	-5.54 $\pm$ 0.53	-8.02 $\pm$ 0.27
<b>6</b>	12.0 $\pm$ 0.3	-6.8 $\pm$ 0.07	0.06 $\pm$ 0.08	-6.7 $\pm$ 0.01
<b>7</b>	38.6 $\pm$ 2.5	-8.14 $\pm$ 0.12	2.11 $\pm$ 0.16	-6.02 $\pm$ 0.04
<b>8</b>	0.398 $\pm$ 0.045	-2.68 $\pm$ 0.12	-6.06 $\pm$ 0.57	-8.74 $\pm$ 0.69
<b>9</b>	0.069 $\pm$ 0.006	-27.97 $\pm$ 0.59	18.2 $\pm$ 0.56	-9.77 $\pm$ 0.05
	1.46 $\pm$ 0.41	-11.23 $\pm$ 0.78	3.26 $\pm$ 0.94	-7.99 $\pm$ 0.16
<b>10</b>	> 1000 <sup>[b]</sup>	-	-	-
<b>11</b>	27.2 $\pm$ 0.99	-10.6 $\pm$ 0.1	4.36 $\pm$ 0.08	-6.24 $\pm$ 0.02
<b>12</b>	NB <sup>[c]</sup>	-	-	-
<b>13</b>	60.6 $\pm$ 14.6	-16.7 $\pm$ 2.0	10.98 $\pm$ 2.14	-5.77 $\pm$ 0.15

[a] Under the experimental conditions used,  $K_d$  values below 100 nM cannot be accurately determined (only  $\Delta H$  could be reliably measured). [b] Binding affinity was too low to accurately determine all of the parameters. [c] NB: No binding was observed or  $K_d$  was too high to allow an accurate determination of the thermodynamic parameters associated with  $Zn^{2+}$  binding. Values shown are averages of triplicate measurements  $\pm$  SD.

This behavior can be rationalized as both compounds contain what appear to be two distinct metal binding sites. The heterocyclic carboxylates **6–8** were found to bind zinc with  $K_d$  values in the low- $\mu\text{M}$  to high-nM range. Finally, compounds **10–13** were found to bind zinc rather weakly with  $K_d$  values for **11** and **13** in the micromolar range, while for L-captopril **10** the  $K_d$  is estimated in the millimolar range and, as for **12**, no appreciable zinc binding could be measured with the ITC conditions used (even at concentration as high as 200  $\mu\text{M}$  in the ITC sample cell). Interesting, the binding of L-captopril to NDM-1 has previously been studied using ITC, revealing a  $K_d$  value of 2.2  $\mu\text{M}$ .<sup>[42]</sup> The low capacity for L-captopril to bind free zinc in solution versus its more effective binding to NDM-1 can be rationalized by the many productive interactions the compound makes within the enzyme active site.<sup>[14]</sup> The results of the ITC investigation were also particularly interesting in the case of compound **12** given that it was found to be the most potent inhibitor in our biochemical assays. These results clearly show that strong binding of free zinc is not a requirement for potent MBL inhibition. Also as noted above, ITC was also used to assess the binding of compounds **1–13** to  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . While for some compounds appreciable binding to calcium and/or magnesium was detected, in all cases this binding was significantly weaker than that measured for  $\text{Zn}^{2+}$  (Tables S1 and S2).

Compounds **1–13** were also evaluated for their ability to synergize with the last-resort carbapenem antibiotic meropenem against a highly resistant (NDM-1 expressing) *E. coli* isolate. Prior to doing so, compounds **1–13** were first tested for any inherent antimicrobial activity against this strain. While compounds **1–13** generally showed no antibacterial activity at clinically relevant concentrations, TPEN **4** did exhibit an MIC of 125  $\mu\text{M}$ . In addition, meropenem was also tested against the same strain confirming its resistance with a measured MIC value of 64  $\mu\text{g}/\text{mL}$  (see Table S3 for full MIC data). The synergy assay performed was designed to establish the concentration of each MBL inhibitor required to reduce the MIC of meropenem by a factor of 4 (i.e., lowering the MIC from 64 to 16  $\mu\text{g}/\text{mL}$ ). In doing so, each MBL inhibitor was administered as a dilution series in combination with meropenem (fixed at 16  $\mu\text{g}/\text{mL}$ ) to establish the lowest inhibitor concentration that resulted in bacterial killing when combined with meropenem at 16  $\mu\text{g}/\text{mL}$ . The results from this assay are summarized in Table 3 and reveal that most of the MBL inhibitors tested do not effectively synergize with meropenem against the highly drug resistant isolate used. Notable exceptions include compounds **2**, **4**, **12**, and **13** which were found to resensitize the NDM-1 expressing isolate to meropenem at 16  $\mu\text{g}/\text{mL}$  at the lowest inhibitor concentration evaluated (15.6  $\mu\text{M}$ ). Using these data we also calculated the fractional inhibitory concentration index value (FICI) of each inhibitor. Bearing in mind that an FICI of  $< 0.5$  indicates synergy, compounds **2**, **4**, **12**, and **13** stand out as the most potent synergizers in this study. As follow up, EDTA **2** and compound **12** were selected for full checkerboard assays with meropenem against *E. coli* RC89, the former as an example of a known zinc stripping inhibitor and the latter discovered through medicinal chemistry efforts. The results show that both

**Table 3.** Synergistic activity data of the MBL inhibitors **1–13**.

Compound	$C_{\text{MIC}/4}$ <sup>[a,b]</sup>	FICI <sup>[c]</sup>
<b>1</b>	250	0.375
<b>2</b>	< 15.6	< 0.258
<b>3</b>	250	0.375
<b>4</b>	< 15.6	< 0.375
<b>5</b>	62.5	0.313
<b>6</b>	500	0.500
<b>7</b>	1000	> 0.500
<b>8</b>	125	0.313
<b>9</b>	> 1000	> 0.500
<b>10</b>	500	0.500
<b>11</b>	500	0.500
<b>12</b>	< 15.6	< 0.258
<b>13</b>	< 15.6	< 0.258

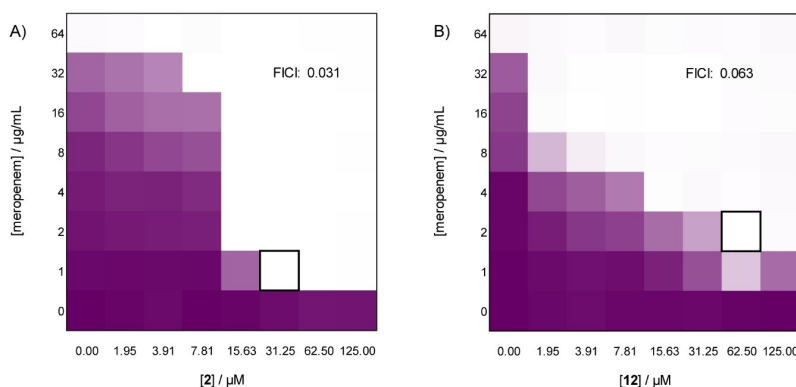
[a] Values shown correspond to  $C_{\text{MIC}/4}$  defined as the lowest concentration of the MBL inhibitor required to achieve a fourfold reduction in the MIC of meropenem. [b] The bacterial isolate used was *E. coli* RC89, an NDM-1 expressing strain obtained from Utrecht Medical Centrum with an MIC for meropenem of 64  $\mu\text{g}/\text{mL}$ . [c] FICI is the fractional inhibitory concentration index where a value of  $< 0.5$  indicates synergy.

compounds display impressive synergy with meropenem, with FICI values of 0.031 and 0.063 for EDTA **2** and compound **12** respectively (Figure 2).

Taken together, the  $\text{IC}_{50}$  values measured, the  $\text{Zn}^{2+}$  binding data, and the results of the antibacterial synergy assays obtained for MBL inhibitors **1–13** reveals some interesting trends. In the case of strong zinc chelators **1–4**, the compounds were found to be much more active against NDM-1 than IMP-1 in the biochemical enzyme inhibition assays. The finding that MBLs of the IMP class are less susceptible to zinc sequestration as a mode of inactivation, while the NDM type are sensitive to strong zinc binders, is in agreement with previous reports.<sup>[11,13,33]</sup> At present, a mechanistic explanation for this selectivity remains elusive. Among inhibitors **1–4**, EDTA **2** and TPEN **4** were also found to effectively synergize with meropenem against a highly resistant NDM-1 producing *E. coli* isolate. It is, however, notable that strong MBL inhibition does not guarantee synergy, as illustrated by DOTA **1** and EGTA **3** both of which were unable to resensitize the same bacteria to meropenem. This may be due to an inability of compounds **1** and **3** to effectively permeate the bacterial cell. The other compound evaluated in this series of chelators, compound **5**, can be viewed as a fragment of **4**. The reduced ability of compound **5** to synergize with meropenem or inhibit NDM-1 and IMP-1 compared with **4** may be attributable to its lower binding affinity for  $\text{Zn}^{2+}$ , although possible differences in membrane permeability may also be a contributing factor in the difference in synergy observed.

The heterocyclic carboxylates **6–8** performed reasonably well in the enzyme inhibition assays. Compounds **6** and **7** were found to be moderate inhibitors of NDM-1 with  $\text{IC}_{50}$  values of 55.0 and 84.0  $\mu\text{M}$ , respectively, but showed no activity toward IMP-1 ( $\text{IC}_{50} > 200 \mu\text{M}$ ). By comparison, DPA (**8**) demonstrated reasonably potent inhibition of both enzymes with an  $\text{IC}_{50}$  value of 4.26  $\mu\text{M}$  for NDM-1 and 15.2  $\mu\text{M}$  for IMP-1. An explanation for these differences is suggested by the result of the ITC-based metal binding assays where DPA (**8**) was found to bind zinc with a  $K_d$  of 398 nM, nearly 100 times lower than the value





**Figure 2.** Checkerboard plots of the full synergy assay for meropenem with A) EDTA **2** and B) inhibitor **12** against meropenem-resistant isolate *E. coli* RC89. The mean optical density of the bacterial growth at 600 nm ( $OD_{600}$ ) is shown as a color gradient, with purple signifying maximum bacterial growth and white as no growth. The combination of inhibitor and antibiotic that gave the lowest FICI is indicated with a black box.

measured for **7** and **30** times lower than **6**. Notably, DPA (**8**) also exhibits appreciable binding to  $Ca^{2+}$  (Table S1), which would likely prove to be detrimental to its effectiveness against MBLs in more complex biological environments containing high levels of free calcium. Inhibitors **6–8** were all found to be rather ineffective at synergizing with meropenem in preventing the growth of the NDM-1 expressing bacterial strain. While compounds **6** and **7** were essentially inactive in the synergy assays, when administered at 125  $\mu$ M the well-studied DPA (**8**) did exhibit the capacity to reduce the MIC of meropenem fourfold against the NDM-1-producing strain.

For thiols **9–11**, a range of activities was observed. In the biochemical assays all three compounds showed moderate inhibition of NDM-1 ( $IC_{50}$  values in the low- $\mu$ M range) while against IMP-1 compound **11** was found to have an  $IC_{50}$  value of 23 nM, making it the most potent inhibitor among all of the compounds evaluated. Interestingly, the metal binding abilities of these compounds have little relation to the inhibitory activity. Dimercaprol **9** is a strong  $Zn^{2+}$  binder with an apparent two-step binding mode ( $K_d$  values in the low-nM to low- $\mu$ M range). Conversely, thiomandelic **11** is a moderate zinc binder with a  $K_d$  of 27.2  $\mu$ M whereas L-captopril **10** exhibited weak binding estimated to be in the millimolar range. In accordance with the biochemical assays, none of these compounds demonstrated particularly strong synergy with meropenem in preventing the growth of the NDM-1 expressing *E. coli* strain.

In comparison, the recently reported MBL inhibitors **12** and **13** were both found to strongly inhibit purified NDM-1 with  $IC_{50}$  values of 5 and 275 nM, respectively – the most potent NDM-1 inhibitors identified among the 13 compounds here studied. Although moderate zinc binding was measured for compound **13** ( $K_d=60.6 \mu$ M), neither **12** nor **13** was found to bind the other metals tested. These findings suggest that these compounds may be less likely to elicit off-target effects arising from promiscuous metal binding. Interestingly, despite being a potent inhibitor of both NDM-1 and IMP-1, compound **12** demonstrated no detectable  $Zn^{2+}$  binding in the ITC assay used. This finding is particularly striking given that potent

inhibition of NDM-1 and other MBLs has generally been associated with compounds that are able to sequester and/or strip zinc from the active site, such as EDTA **2**. It is, however, clear from our ITC studies that this is not the mode of MBL inhibition for compound **12**. These findings are even more notable given that **12** and **13** were also found to generally outperform known zinc sequestering MBL inhibitors in the synergy assay performed. Unlike most of the other inhibitors in this assay, **12** and **13** were found to be very effective in synergizing with meropenem against the NDM-1 expressing strain used. It is worth noting that **12** and **13** are the products of dedicated screening and optimization efforts aimed at identifying novel MBL inhibitors. Our findings reveal that there is indeed likely to be value in pursuing such focused approaches in the development of MBL inhibitors designed to specifically interact with the enzyme active site rather than relying explicitly on strong metal binding.

As noted above, a recently published study by the Crowder group reported the use of different biochemical and biophysical approaches to evaluate the inhibitory mechanism of a panel of MBL inhibitors.<sup>[31]</sup> Notably, they introduced the use of native mass spectrometry to evaluate the binding of inhibitors to MBL enzymes. This elegant method requires low concentrations of enzymes and inhibitors (at low- to sub- $\mu$ M levels) which are also closer to physiological conditions. Using this approach, they found that EDTA effectively strips zinc from NDM-1 whereas L-captopril forms a ternary complex. Also notable are the Crowder group's findings with compound **13** and the effect it has on NDM-1 and VIM-2. Equilibrium dialysis experiments using both enzymes showed that like L-captopril, compound **13** does not remove zinc from NDM-1 and VIM-2. In contrast, incubation with EDTA resulted in substantial depletion of zinc from NDM-1 and VIM-2. Interestingly, our ITC experiments provide additional clarification here as we measured only moderate zinc binding affinities for both L-captopril **10** and compound **13**, both of which showed  $K_d$  values in the micro- to millimolar range, while that of EDTA is estimated in the sub-nM range. In this regard, the use of ITC to quantify metal binding

affinity presents an attractive approach to compliments the panel of biochemical and biophysical techniques applied by Crowder and co-workers in investigating the mechanism of MBL inhibitors.<sup>[42]</sup> Notably, ITC offers a means to conveniently assess potentially problematic promiscuous metal binding by MBL inhibitor candidates. Furthermore, the trend observed in our present study, indicates that inhibitors that display an extremely high affinity to bind zinc ( $K_d$  at the low-nM level or below) are unlikely to form a ternary complex with the MBL enzymes.

## Conclusion

In recent years a variety of compounds have been described as possessing MBL inhibitory activity. However, in many cases suboptimal selectivity (as for chelating agents) or stability (as for thiols) limits their potential for drug development. Moreover, active-site heterogeneity among MBLs, as manifested in the differing sensitivities of NDM-1 and IMP-1 towards the chelating agents investigated in this study, points to challenges in the development of a broad-spectrum MBL inhibitor. As our investigations further reveal, the use of different biochemical and biophysical methods allows for a more complete understanding of the modes of action of MBL inhibitors. Such mechanistic insights are likely to be of importance in anticipating issues relating to *in vivo* safety and selectivity for MBL inhibitors in the early phases of drug development. Among the MBL inhibitors evaluated in our study, the indole carboxylate derivative **12** was found to exhibit an impressive combination of activity and selectivity. The compound effectively resensitizes MBL-expressing bacteria to meropenem and is a potent and broad-spectrum MBL inhibitor with nanomolar  $IC_{50}$  values against both NDM-1 and IMP-1. Furthermore, compound **12** shows no appreciable binding to free  $Zn^{2+}$ ,  $Ca^{2+}$ , or  $Mg^{2+}$ . Taken together, these findings suggest that compound **12** and its analogues may present promising candidates for future drug development efforts aimed at overcoming MBL-expressing pathogens.

## Experimental Section

### Materials

Commonly used solvents and reagents were purchased from either Sigma-Aldrich or Combi-blocks. Compounds **1**, **2** and **5** were purchased from Sigma Aldrich. Compounds **6**, **7** and **8** were purchased from Combi-blocks. Compounds **3** and **9** were purchased from Alfa Aesar, compound **4** from Cayman Chemicals, and **10** from Acros. Compound **11** was prepared as previously described.<sup>[18]</sup>

### Compound synthesis

Compounds **12** and **13** were prepared according to published patent procedures with minor adjustments.<sup>[28,29]</sup> Full experimental details of the synthetic procedures used for the preparation of **12** and **13** are provided in the Supporting Information.

## Enzyme expression and purification

The plasmids containing NDM-1 and IMP-1 were provided by Prof. Christopher Schofield (Oxford University). To obtain IMP-1,<sup>[43]</sup> *E. coli* BL21(DE3) pLysS transformed with the plasmid was grown at 37 °C in 2 L of lysogeny broth (LB) medium supplemented with kanamycin (50 µg/mL) to ensure selective growth. When the culture reached an optical density ( $OD_{600\text{ nm}}$ ) of 0.6, protein production was induced using 0.5 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG). The cultures were grown for another 4 h before the cells were harvested by centrifugation (4000 g) for 20 min and resuspended in 50 mM HEPES pH 7.0, 250 mM NaCl. The cells were lysed using sonication and centrifuged for 30 min at 30000g. The supernatant was diluted with buffer A (50 mM HEPES pH 7.0, 100 µM  $ZnSO_4$ ) before being loaded onto a 1 mL HiTrap SP FF column (GE Healthcare) and subsequently eluted using a gradient of buffer B (50 mM HEPES pH 7.0, 100 µM  $ZnSO_4$ , 1 M NaCl). The fractions were run on a SDS-PAGE gel and those containing IMP-1 were combined, concentrated using spin filter columns (Millipore) and loaded onto a size-exchange column (Superdex75 16/60; GE Healthcare). The fractions were once again assessed by SDS-PAGE and the pure fractions were combined and concentrated. The concentration of the protein was determined using a NanoDrop spectrometer. The expression and purification of NDM-1 was performed as previously described.<sup>[44]</sup>

## Enzyme inhibition assays

The half-maximal inhibitory concentration ( $IC_{50}$ ) of each compound was determined against NDM-1 and IMP-1. The compounds were serially diluted (50 µL) and incubated with 25 µL of NDM-1 (40 pM) or IMP-1 (125 pM) for 15 min. Upon addition of FC5 substrate (0.5 µM for NDM-1 and 20 µM for IMP-1, 25 µL in each case), fluorescence was monitored over 25 cycles ( $\lambda_{ex}$  = 380 nm,  $\lambda_{em}$  = 460 nm) on a Tecan Spark plate reader. The initial velocity data was used to produce the  $IC_{50}$  curves using GraphPad prism 7 software. The buffer used in the experiments was 50 mM HEPES pH 7.2 containing 0.01% Triton X-100 and 1 µM zinc sulfate, and the microplates used were µClear®, black half-area 96-well plate (Greiner Bio-one).

## Isothermal titration calorimetry

ITC experiments were performed using a MicroCal PEAQ-ITC Automated instrument (Malvern). The test compounds and metal salts were dissolved in Tris-HCl buffer (20 mM, pH 7.0). The solutions of divalent cations (prepared from  $ZnSO_4$ ,  $CaCl_2$ , and  $MgCl_2$ ) were titrated into a solution of the compound of interest in 19 aliquots of 2 µL (except the first injection, which was 0.4 µL) with 150 s between injection. The only exceptions were dipicolylamine **5** and dimercaprol **9**, for which 32 and 28 aliquots of 1 µL (except the first injection of 0.4 µL) were titrated with 100 s between injections. The concentration of cations vs small molecules (ligands) for each titration is listed under each thermogram in Figure S2. All the experiments were performed at 25 °C in triplicate with the reference power set to 10 µcal/s. The data generated was analyzed using the MicroCal PEAQ-ITC Analysis Software.

## Antibacterial activity

Determination of minimum inhibitory concentration (MIC). Antibacterial assays were performed according to clinical and laboratory standards institute (CLSI) guidelines. Test compounds were serially diluted with Mueller-Hinton broth (MHB) in polypropylene 96-well plates (50 µL in each well). *E. coli* RC0089 (NDM-1) was



inoculated into tryptic soy broth (TSB) and incubated at 37 °C. Once the bacteria cells grew to an OD<sub>600</sub> of 0.5, the suspension was diluted with MHB (final concentration 10<sup>6</sup> CFU/mL) and then added to the microplates containing the test compounds (50 µL to each well, final volume: 100 µL). After incubation at 37 °C for 16–20 h, the microplates were inspected for growth inhibition. MIC values were defined as the lowest concentration of the compound that prevented visible growth of bacteria.

**Synergy assay (C<sub>MIC/4</sub>).** The test compounds were serially diluted with MHB starting from a maximum concentration of 1000 µM (25 µL in each well). Meropenem (25 µL) was then added to the wells to achieve a final concentration of 16 µg/mL. *E. coli* RC0089 (NDM-1) was cultured and added to the microplates as described above (50 µL to each well, final volume: 100 µL). The C<sub>MIC/4</sub> value was defined as the lowest concentration of the inhibitor that prevented the visible growth of the bacteria when combined with meropenem at 1/4 of its MIC. FICI values were established by applying the following formula where an FICI < 0.5 indicates synergy:

$$FICI = \frac{MIC_{\text{Meropenem in combination}}}{MIC_{\text{Meropenem alone}}} + \frac{MIC_{\text{Inhibitor in combination}}}{MIC_{\text{Inhibitor alone}}}$$

**OD<sub>600</sub> checkerboard assay.** The test compounds were serially diluted with MHB starting from a maximum concentration of 125 µM (25 µL in each well). Meropenem was serially diluted to 4x the final concentration before being added to the test compounds (25 µL). *E. coli* RC0089 was cultured and added to the microplates as described above (50 µL to each well, final volume: 100 µL). The microplates were then incubated at 37 °C with shaking. After 18 h, the optical density of each well was measured using a Tecan Spark plate reader at 600 nm.

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## Conflict of Interest

The authors declare no conflict of interest.

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