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Small Molecule Carboxylates Inhibit Metallo- β -lactamases and Resensitize Carbapenem-Resistant Bacteria to Meropenem

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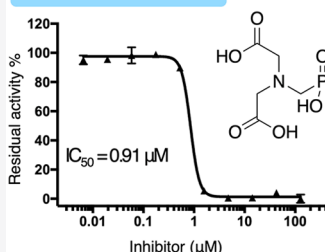
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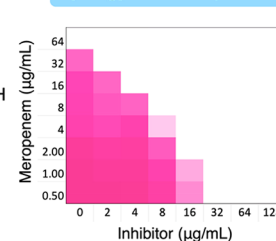
ABSTRACT: In the search for new inhibitors of bacterial metallo- β -lactamases (MBLs), a series of commonly used small molecule carboxylic acid derivatives were evaluated for their ability to inhibit New Delhi metallo- β -lactamase (NDM)-, Verona integron-encoded metallo- β -lactamase (VIM)-, and imipenemase (IMP)-type enzymes. Nitrotriacetic acid (**3**) and *N*-(phosphonomethyl)-iminodiacetic acid (**5**) showed promising activity especially against NDM-1 and VIM-2 with IC_{50} values in the low-to-sub μ M range. Binding assays using isothermal titration calorimetry reveal that **3** and **5** bind zinc with high affinity with dissociation constant (K_d) values of 121 and 56 nM, respectively. The *in vitro* biological activity of **3** and **5** against *E. coli* expressing NDM-1 was evaluated in checkerboard format, demonstrating a strong synergistic relationship for both compounds when combined with Meropenem. Compounds **3** and **5** were then tested against 35 pathogenic strains expressing MBLs of the NDM, VIM, or IMP classes. Notably, when combined with Meropenem, compounds **3** and **5** were found to lower the minimum inhibitory concentration (MIC) of Meropenem up to 128-fold against strains producing NDM- and VIM-type enzymes.

KEYWORDS: NDM-1, MBL inhibitors, zinc binding, isothermal titration calorimetry, synergy

Potent NDM-1 Inhibition



Synergy with Meropenem



Antibiotic resistance threatens to reduce the efficacy of currently available antibiotics and places a substantial burden on global health and the world economy.^{1,2} Resistance to β -lactam antibiotics can be caused by a diverse group of enzymes known as β -lactamases. While based on sequence homology, these enzymes are categorized into classes A–D (known as Ambler classification),³ mechanistically they are classified as serine- β -lactamases (SBLs, Ambler classes A, C, and D) or metallo- β -lactamases (MBLs, Ambler class B).⁴ SBLs inactivate β -lactams via the hydrolytic action of a nucleophilic serine in their active site. First-generation SBL inhibitors, including clavulanic acid, sulbactam, and tazobactam as well as the more recently approved avibactam and vaborbactam, are available to rescue β -lactams in the presence of SBL-producing bacteria.^{5,6} MBLs on the other hand are metallo-enzymes that hydrolyze β -lactams by the action of a zinc-activated nucleophilic water molecule that is formed in the active site. To date, there are no FDA-approved MBL inhibitors available. Of particular concern are the clinically important MBLs including the New Delhi metallo- β -lactamase (NDM), VIM, and IMP families that possess carbapenemase activity,⁷ adding further urgency to the development of MBL inhibitors to combat MBL-producing bacterial infections.

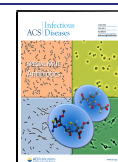
Small molecules with the ability to inhibit MBLs have been the topic of a number of comprehensive reviews.^{8–11} The

majority of the known MBL inhibitors contain functional groups that can bind zinc. In this regard, the most common small molecules possessing anti-MBL activity are thiol-containing compounds,^{12–15} sulfonylhydrazones,¹⁶ bis-carboxylic acids,^{17,18} picolinic acids,^{19,20} and commonly used chelating agents,^{21,22} including their bacteria-targeting analogs.^{23,24} As an example, the natural product aspergillomarasmine A (AMA) was recently identified by Wright and co-workers who screened fungal extracts for anti-MBL activity. AMA was shown to be a potent inhibitor of both NDM- and VIM-type enzymes and importantly displays *in vivo* efficacy.²⁵ Also of interest are the recently developed cyclic boronate SBL- and MBL-inhibitors, which mimic the tetrahedral intermediate formed upon the nucleophilic attack of a serine-hydroxyl group (SBLs) or zinc-bound water molecule (MBLs) at the β -lactam unit.^{26–30} In addition, reports have also described compounds with alternative modes of MBL inhibition including covalent inhibitors^{31–33} and DNA

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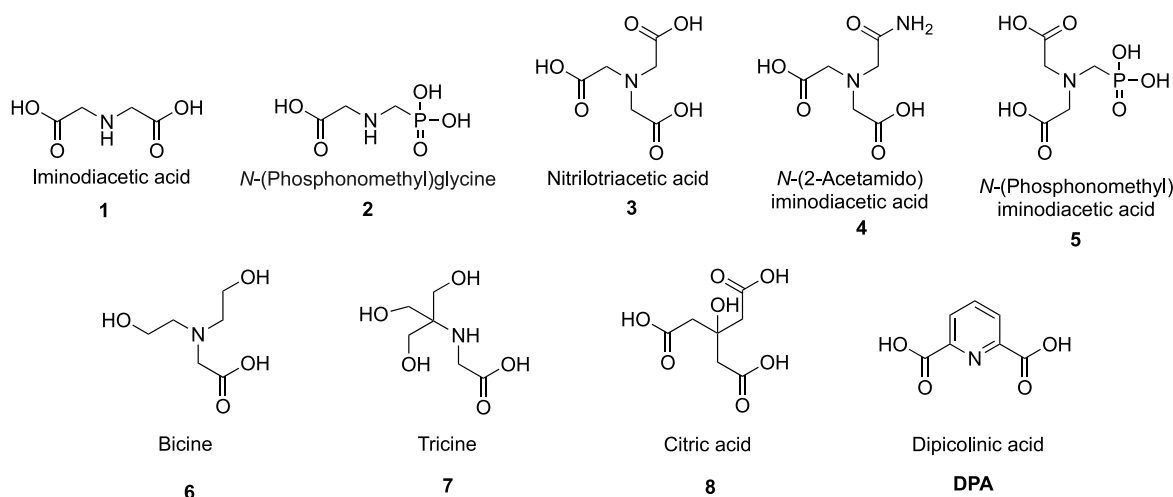


Figure 1. Small molecule carboxylic acids as potential MBL inhibitors.

aptamers proposed to operate via allosteric mechanisms of inhibition.³⁴

In reviewing the literature, we noted that sulfonic acid buffer components such as MES and PIPES have previously been reported to be weak MBL inhibitors.³⁵ This prompted us to investigate the possibility of identifying new MBL inhibitor candidates among other commonly used small molecule buffer components containing multiple carboxylic acid and/or phosphonate functionalities. Given that zinc binding is a key aspect of the mechanism of action for a majority of MBL inhibitors, we specifically focused our attention on common buffer reagents and structurally related small molecules reported to interact with metals (Figure 1).

The panel of small molecules shown in Figure 1 were first screened for their inhibitory activity against purified MBLs including NDM-1, VIM-2, and IMP-28. The substrate used for the enzyme inhibition assay was a fluorescent cephalosporin derivative developed by Schofield and co-workers for assessing MBL activity.³⁶ As shown in Table 1, nitrilotriacetic acid (NTA, 3) and its bioisosteres (4; 5, N-(phosphonomethyl)iminodiacetic acid) showed promising activity against NDM-1 and VIM-2, superior to that of dipicolinic acid (DPA), a well-studied MBL inhibitor.^{19,20} Notably, the much weaker inhibitory activity of the disubstituted analogs 1 and 2 point

Table 1. IC₅₀ Values Determined against NDM-1, VIM-2, and IMP-28

compound	IC ₅₀ (μM) ^a		
	NDM-1	VIM-2	IMP-28
1	>200	>200	>200
2	75 ± 2	41 ± 6	>200
3	1.3 ± 0.07	2.4 ^b	112 ± 3
4	2.3 ± 0.05	25 ^b	>200
5	0.91 ± 0.05	0.68 ± 0.02	39 ± 7
6	>200	>200	>200
7	>200	>200	>200
8	132 ± 15	102 ± 7	>200
DPA	3.8 ± 0.04	2.9 ± 0.5	17 ± 1

^aValues reported as mean ± SD of at least 3 independent experiments. ^bDue to the complex shape of the log[concentration] – activity plot, accurate fitting was not possible; the reported values are therefore an estimation (see the Supporting Information).

to the necessity of three carboxyl(phosphoryl) substituents in order to achieve potent inhibition of NDM-1 and VIM-2, most probably by tightly chelating zinc ions. Interestingly, compounds 1–8 all exhibited little-to-no activity against IMP-28. This observation is in line with previous investigations that have found the IMP class of MBLs to be less sensitive to inhibition by zinc-binding agents.^{25,37} To establish whether the inhibition measured was time dependent, the IC₅₀ values of compounds 3, 5, and DPA for NDM-1 were also determined after preincubating the inhibitor and enzyme for various times including 0, 10, 20, 40, and 60 min, as previously described for a different class of NDM-1 inhibitors.³⁸ As shown in Figure S3, preincubation time does not significantly affect the potency of the tested compounds under the assay conditions.

The majority of MBL inhibitors fall into one of two groups: those that interact with zinc as part of their binding in the MBL active site forming a ternary complex or those that actively strip zinc from the MBL active site driven by their strong chelating ability.^{39,40} Captopril is an example for the former, while known chelating agents such as EDTA and AMA represent the latter.^{19,41} In determining the IC₅₀ value of N-(phosphonomethyl)iminodiacetic acid 5 against NDM-1, it was noted that, in the presence of different concentrations of zinc sulfate (ranging from 0.1 μM to 20 μM), the IC₅₀ values measured also changed, revealing a zinc-dependent effect similar to that for DPA. In comparison, and as expected, the inhibitory activity of captopril is not influenced by varying the concentration of exogenous zinc added to the assay media (Figure 2). These findings support a zinc-sequestration based mechanism of NDM-1 inhibition for compound 5.

Further evidence for high affinity zinc binding by compound 5 was obtained by the use of isothermal titration calorimetry (ITC). As we have previously demonstrated for thiol based small molecule zinc-binding MBL inhibitors,¹³ ITC allows for the direct determination of the dissociation constant (*K*_d) as well as the thermodynamic parameters Δ*G*, Δ*H*, and Δ*S*. Among the small molecules tested as part of the current study, compounds 3–5 were found to be strong zinc binders with *K*_d values of 121, 231, and 56 nM, respectively (Table 2). Interestingly, the affinity of compounds 3–5 for other biologically relevant divalent cations like Ca²⁺ and Mg²⁺ was negligible by ITC with binding interactions too weak to allow for an accurate determination of thermodynamic parameters.

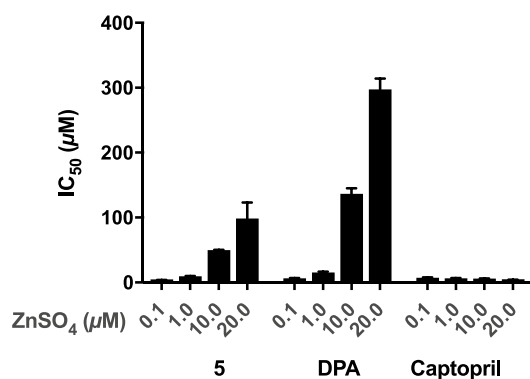


Figure 2. Effect of zinc on the inhibitory activity of compound 5, DPA, and captopril against NDM-1.

Previous reports have also described potentiometric titration^{42–44} and ITC based methods for studying the metal binding properties of related compounds.^{45–47} It should be noted that, in these earlier studies, the associated K_d values measured for the binding of Ca^{2+} and Zn^{2+} by DPA were somewhat lower than the values obtained in our investigations, an effect we ascribe to differences in the buffers used. Specifically, given the buffering capacity of the test compounds evaluated in our study, we chose to employ 100 mM Tris buffers to avoid any pH mismatch. Notably, our ITC data reveal a strong correlation between these compounds' capacity to inhibit MBL activity and their zinc binding ability (see Table S1 for the full ITC data).

The results of our investigations, as well as other recently published studies, indicate that the incorporation of the phosphonic acid moiety is a promising approach in designing potent MBL inhibitors.^{48–50} In line with our findings related to the enhanced potency of compound 5 relative to compound 3 are recent studies showing that phosphonic acid analogues of picolinic acid demonstrate increased potency against NDM-1.^{20,49} In addition, phosphonate analogues of the well-known mercapto-carboxylic acid MBL inhibitors (represented by thiomandelic acid) demonstrate enhanced inhibitory activity.⁴⁸ In light of our findings and the studies mentioned above, the incorporation of a phosphonic acid moiety into the structures of other MBL inhibitors such as cyclic boronates (exemplified by VNRX-5133)³⁰ may also provide access to new classes of hybrid MBL inhibitors.

The ability of compounds 1–8 to restore the activity of Meropenem, a last resort carbapenem, against a representative MBL-expressing strain was evaluated using a clinical NDM-1 positive isolate (coded *E. coli* RC0089). Using a checkerboard assay, multiple concentration combinations of MBL inhibitor + Meropenem were tested, allowing for the calculation of the fractional inhibitory concentration (FIC) index according to the following expression (where an FIC index (FICI) of <0.5 indicates a synergistic relationship):

$$\text{FICI} = \frac{\text{MIC of Meropenem in combination}}{\text{MIC of Meropenem alone}} + \frac{\text{MIC of MBL inhibitor in combination}}{\text{MIC of MBL inhibitor alone}}$$

Among the compounds tested, 3–5 showed a synergistic relationship with Meropenem with compound 5 demonstrating the highest potency with the lowest FIC index of 0.047 (Figure 3). Compounds 3 and 5 were both very effective in restoring the activity of Meropenem against the NDM-1 producing *E. coli* strain used in the initial screen and were therefore also tested in combination with Meropenem against a larger panel of 38 Gram-negative clinical isolates displaying carbapenem resistance (Table S2). While compounds 3 and 5 exhibited no antibacterial activity at the highest tested concentration of 256 $\mu\text{g}/\text{mL}$, both were found to effectively enhance the activity of Meropenem against strains expressing NDM- and VIM-type enzymes. When administered at a concentration of 32 $\mu\text{g}/\text{mL}$, both 3 and 5 reduced the minimum inhibitory concentration (MIC) of Meropenem by up to 128-fold against these strains, a synergism equivalent to or better than that observed for DPA. Overall, compound 5 reduced the MIC of Meropenem to its clinically susceptible concentration ($\leq 1 \mu\text{g}/\text{mL}$) for 67% of the NDM- and VIM-type producing isolates tested, while for compound 3 and DPA, this ratio was 37% and 53%, respectively. In comparison, when tested against strains expressing IMP-type enzymes, the synergistic activity of 3 and 5 was modest, leading to no more than a 4-fold reduction of MIC in most cases, a trend also mirrored for DPA. In addition, the complete lack of synergy observed against strains expressing serine-carbapenemases such as KPC-2 and OXA-48 further demonstrates the inhibitory activities of compounds 3 and 5 to be MBL-specific. Also, among the bacterial species screened, *P. aeruginosa* proved to be more resistant to the synergistic combinations tested. This is apparent when comparing the antibacterial activities of the combinations against NDM-1 and VIM-2 producing *P. aeruginosa* isolates versus the corresponding *E. coli* and *K. pneumoniae* counterparts (see Table S2).

CONCLUSION

The clinically most relevant MBLs continue to be the NDM, VIM, and IMP classes and present a significant challenge to the efficacy of virtually all classes of β -lactam antibiotics including “last-line-of-defense” carbapenems such as Meropenem. Despite this, no inhibitors are clinically available to combat resistant infections caused by Gram-negative pathogens that express MBLs. The current study expands our understanding of the diversity of small molecule carboxylic acids that inhibit MBLs and synergize with carbapenems. By screening a series of available and commonly used small molecule carboxylates, we found that nitrilotriacetic acid (3) and its phosphoric acid analogue *N*-(phosphonomethyl)iminodiacetic acid (5) are both potent inhibitors of NDM- and VIM-type enzymes with sub- to low- μM IC_{50} values. Using ITC, both 3 and 5 were

Table 2. ITC Based Thermodynamic Parameters for the Binding of Zinc by Compounds 3–5

compound	K_d (nM)	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)	ΔG (kcal/mol)
3	121 \pm 7	−4.890 \pm 0.219	−4.546 \pm 0.248	−9.400 \pm 0.042
4	231 \pm 10	−2.957 \pm 0.069	−6.100 \pm 0.079	−9.057 \pm 0.026
5	56 \pm 15	−3.077 \pm 0.113	−6.837 \pm 0.274	−9.910 \pm 0.155

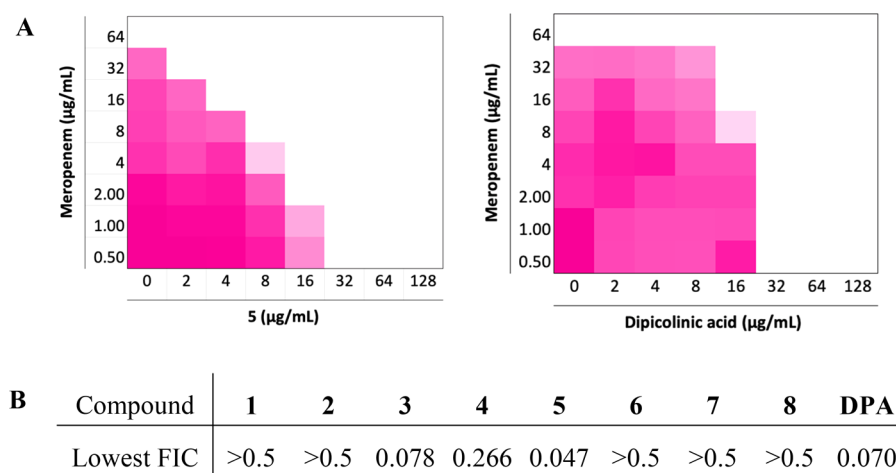


Figure 3. (A) Checkerboard plots for compound 5 and DPA in combination with Meropenem tested against an NDM-1 producing strain of *E. coli*. The optical density of the bacteria at 600 nm (OD_{600}) has been shown as a color gradient between white (no bacterial growth) and magenta (maximum growth). (B) The lowest FIC values calculated for compounds 1–8.

shown to bind zinc with nanomolar affinity. When further tested against a broad panel of MBL-producing Gram-negative pathogens, compounds 3 and 5 effectively reduced the MIC of Meropenem against NDM- and VIM-type enzymes. As for the well-characterized MBL inhibitors DPA and AMA, the mechanism of inhibition for 3 and 5 appears to be largely driven by zinc-sequestration. While AMA was reported to have *in vivo* efficacy,²⁵ it is unlikely that strong zinc-binding small molecule carboxylates like 3 and 5 are suitable as clinical candidates. Rather, such compounds represent readily available inhibitors for biochemical studies of MBLs. Furthermore, given their small size and structural simplicity, such compounds may serve as leads for further optimization. One approach may be to administer such compounds as prodrugs that are activated only upon entry to the bacterial cell. In the absence of clinically approved MBL-inhibitors and with increasing rates of MBL-driven carbapenem resistance, it is important that many approaches, including unconventional avenues, be explored in the pursuit of an effective therapeutic response.

METHODS

Enzyme Production and Purification. The plasmid constructs of NDM-1 and VIM-2 were a kind gift from Prof. Christopher J. Schofield (Oxford University). The IMP-28 construct was designed in the pET28b vector with a C-terminal His-tag. The corresponding enzymes were expressed and purified as described in the [Supporting Information](#).

IC₅₀ and Zinc Dependency Assay. All the test compounds were dissolved and serially diluted in 50 mM HEPES, pH 7.2, supplemented with 0.01% triton X-100 and 1 μ M ZnSO₄. The MBL enzymes (60 pM NDM-1, 100 pM VIM-2, and 60 pM IMP-28) were then added to the wells and incubated at 25 °C for 15 min. Next, the fluorescent cephalosporin substrate FCS³⁶ (0.5 μ M for NDM-1 and VIM-2, 16 μ M for IMP-28) was added to the wells, and the fluorescence was monitored immediately over 30–40 scanning cycles (λ_{ex} 380 nm, λ_{em} 460 nm) on a Tecan Spark plate reader. Using the initial velocity data plotted against inhibitor concentration, the half-maximal inhibitory concentrations were calculated by an IC₅₀ curve-fitting model in GraphPad Prism 7 software. 2,6-Dipicolinic acid was used as the positive control. The IC₅₀ of captopril, dipicolinic acid, and compound 5 was

also evaluated in the presence of different concentrations of zinc sulfate (0.1, 1, 10, and 20 μ M) against NDM-1, following the procedure described above.

Isothermal Titration Calorimetry (ITC). The test compounds were evaluated for their ability to bind zinc using an automated PEAQ-ITC calorimeter (Malvern). Zinc sulfate dissolved in 100 mM Tris (pH 7.0) was titrated into the test compounds dissolved in the same buffer over 19 \times 2 μ L aliquots (except for the first aliquot, which was 0.4 μ L). The titrations were performed at 25 °C, and reference power was set at 10 μ cal/s. Peak integration and curve-fitting was done using the PEAQ-ITC data analysis software provided by the manufacturer. The blank titrations included buffer titrated in the test compounds and zinc sulfate titrated in buffer, both of which showed negligible signals attributed to the heat of dilution (see the [Supporting Information](#) for the thermograms).

Antibacterial Assays. All antibacterial assays were carried out following the guidelines published by the Clinical and Laboratory Standards Institute (CLSI). Bacterial strains and clinical isolates were cultured on blood agar and incubated overnight at 37 °C. Fresh colonies were suspended in tryptic soy broth (TSB) and incubated at 37 °C with shaking. Following growth to the exponential phase (OD_{600} = 0.5), the bacterial suspension was diluted to 10⁶ CFU/mL in Mueller-Hinton broth (MHB) and added to the test compounds prepared as described for each assay.

Single Concentration Synergy Assay. On a polypropylene microplate, Meropenem was dissolved and serially diluted in MHB (25 μ L/well). Compounds 3, 5, and DPA with a final concentration of 32 μ g/mL (25 μ L/well) were then added to the wells. Following the addition of the diluted bacterial suspensions prepared as described above (50 μ L/well), the microplates were incubated at 37 °C with shaking, and after 16–20 h, the plates were inspected for the bacterial growth. Minimum inhibitory concentration (MIC) values were reported as the lowest concentration of the antibiotic/test compounds that prevents the visible growth of bacteria. All the assays were performed in triplicate, and the median values were used to report MICs.

OD₆₀₀ Checkerboard Assay. Meropenem was dissolved and serially diluted on the polypropylene microplates in MHB (25

$\mu\text{L}/\text{well}$). The test compounds dissolved and serially diluted to the final concentration ranging from 128 to 1 $\mu\text{g}/\text{mL}$ were then added to Meropenem (25 $\mu\text{L}/\text{well}$). *E. coli* RC0089, a clinical isolate producing NDM-1, grown to the exponential phase and diluted in MHB was added to the microplate (50 $\mu\text{L}/\text{well}$), which was then incubated at 37 °C with shaking. After 16–20 h, the optical density of the wells was scanned at 600 nm on a Tecan Spark plate reader.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsinfecdis.9b00459>.

Procedure for enzyme production and purification, IC₅₀ curves, time-course of NDM-1 inhibition by 3, 5, and DPA, thermodynamic data and ITC thermograms, checkerboard synergy graphs, and MIC data against clinical isolates (PDF)

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Notes

The authors declare no competing financial interest.

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