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Crystallographic Analyses of Isoquinoline Complexes Reveal a New Mode of Metallo- β -Lactamase Inhibition

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Abstract

Crystallographic analyses of the VIM-5 metallo- β -lactamase (MBL) with isoquinoline inhibitors reveal non zinc ion binding modes. Comparison with other MBL-inhibitor structures directed addition of a zinc-binding thiol enabling identification of potent B1 MBL inhibitors. The inhibitors potentiate meropenem activity against clinical isolates harboring MBLs.

Graphical Abstract

Crystallographic analyses revealed new metallo- β -lactamase inhibition modes and enable the design of potent zinc-binding inhibitors.

The global spread of antibacterial resistance mediated by metallo- β -lactamases (MBLs), which catalyse hydrolysis of almost all β -lactam antibiotics¹, is an increasing clinical problem.^{2,3} β -Lactamases are divided into classes A, B, C, and D; Class A, C, and D use a

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nucleophilic serine in catalysis, whereas class B uses one or two zinc ions in catalysis (Fig. 1).^{1, 4} Serine β -lactamase inhibitors are used clinically in combination with a penicillin or cephalosporin.^{5–7} No clinically useful MBL inhibitors have been reported, in part because the prevalence of MBL resistance has been limited until recently. Most reported MBL inhibitors work via active site metal chelation.^{6, 8–13} We are interested in identifying new types of MBL inhibitors¹⁴, which will enable the breadth of activity required for clinical application, whilst not inhibiting structurally related human MBL-fold enzymes^{15, 16}. The aim of obtaining the breadth of MBL selectivity is complicated by structural variations in the B1 MBL active site loops, as observed in crystal structures of the Verona Integron-encoded MBL (VIM), Imipenemase (IMP), and New Delhi (NDM) B1 MBLs.^{17–19}

We recently reported studies comparing the properties of VIM variants.²⁰ The results reveal minor differences in the kinetic parameters of the tested variant/substrate combinations and in the protein folds. However, differences in in-vitro inhibition profiles were observed which may be relevant to inhibitor development. In particular, using a fluorescence-based assay, we observed differences in potencies for enantiomers of tryptophan-isoquinoline linked inhibitors against VIM variants (**1** and **2**, Fig. 2a).²¹ The (*R*)-enantiomer (**1**) was more potent than the (*S*)-enantiomer (**2**) for the tested VIM variants (VIM-5, VIM-2, VIM-4, and VIM-38), whereas the (*R*)-enantiomer (**1**) was selective for VIM-5 and VIM-38 over VIM-1 and VIM-4 (Table S1).²⁰ Both **1** and **2** manifested inhibition (albeit modest) of São Paulo metallo- β -lactamase (SPM)-1, IMP-1, NDM-1, and VIM-2.²¹ Nevertheless, the isoquinoline based inhibitors show potential for broad-spectrum MBL inhibition. To investigate the mode of action of **1** and **2** against MBLs, we performed crystallographic analyses on VIM-5. The results reveal a new mode of MBL inhibition and enabled the design of modified thiol-based zinc binding compounds leading to more potent inhibitors.

We obtained crystal structures of VIM-5 in complex with **1** and **2** to high resolution; VIM-5:**1** and VIM-5:**2** structures were obtained by co-crystallization (Table S2) and diffracted to 1.96 Å and 1.99 Å resolution, respectively. The VIM-5:**1** structure crystallised with two molecules in the asymmetric unit (space group $P2_1 2_1 2_1$); the VIM-5:**2** structure contains three molecules in the asymmetric unit (space group $F2 2 2$) (Table S3). In both structures, there was clear $F_o - F_c$ density in the VIM-5 active site, into which **1** and **2** could be confidently modelled (Fig. S1).

In the VIM-5:**2** structure, oxidation of Cys221 to a sulphinic acid (Csd221) prevented binding of Zn2 (Fig. 2c); we attempted co-crystallization/soaking, but failed to obtain a di-zinc VIM-5 structure complexed with **2**. Crystallisation of this crystal form may have been due to time dependent Cys221 oxidation, since crystals took several weeks to appear. Superimposition of the mono-zinc VIM-5:**2** structure with the di-zinc VIM-5 structure without ligand (PDB ID: 5A87)²⁰ reveals, apart from the different number of bound zinc ions, a similar fold and active site except for small changes in the immediate environment around the oxidised and unoxidised Cys221 (Fig. S3).

We also obtained a structure of VIM-5 complexed with **1** in which Cys221 was oxidised (1.05 Å resolution). This structure revealed a near identical binding mode for **1** as observed in the unoxidised di-zinc VIM-5:**1** structure (Fig. S4 and S5). Thus, it appears likely that

Cys221 oxidation and loss of one of the two zinc ions from the active site of VIM-5 has a limited effect on the binding mode of **1** and, by inference, **2**.

In the VIM-5:**1** complex, **1** binds close, but not directly, to the active site zinc ions and is positioned to interact with the catalytically important Arg228 and Tyr67 (using standard BBL MBL numbering²²) (Fig. 2b). The carboxylate of **1** is positioned to make hydrogen-bond and electrostatic interactions with Ser227, Arg228, Thr229, and Ser230 on the L10 loop, and its isoquinoline ring is positioned to π - π stack with Tyr67 on the L3 loop and make cation- π interactions with Arg228 (Fig. 2b and Fig. S2a). Similarly to the binding of **1**, **2** binds adjacent to, but not directly with, the active site zinc ions (Fig. 2c); its carboxylate is positioned to form hydrogen bonds with Arg228 and Asn233 on the L10 loop (similar to the carboxylate of β -lactam substrates), and its isoquinoline and indole moieties are positioned to make π - π stacking interactions with Phe61 and Try67 on the L3 loop, respectively (Fig. 2c and Fig. S2b).

The crystallographic analyses of **1** and **2** with VIM-5 reveal an inhibition mode that does not involve direct zinc chelation. To validate this mode, we used ¹H Carr-Purcell-Meiboom-Gill (CPMG) and ligand-observe NMR (water-ligand observed gradient spectroscopy, wLOGSY) to test for binding to di-Zn(II) and *apo*-VIM-5.¹⁴ The latter was catalytically inert and generated by incubation of di-Zn(II)-VIM-5 with ethylenediaminetetraacetic acid (EDTA) (Fig. S6). The results reveal **1** and **2** bind strongly to both *apo*-VIM-5 (Fig. 3a–b, S7a–b) and di-Zn(II)-VIM-5 (K_D values for **1** and **2**, 0.8 μ M and 1.03 μ M, respectively, (using racemic thiomandelic acid as a reporter molecule Fig. S9a–b)²⁰). By contrast, thiomandelic acid, a broad-spectrum MBL inhibitor²³, does not bind to *apo*-VIM-5 (Fig. 3c, S7c, S8); K_D for thiomandelic acid with di-Zn(II)-VIM-5, 170 nM (Fig. S9c). Thus, zinc ions are not required for **1** and **2** to bind to VIM-5 in solution, consistent with the binding mode observed in the crystal structures.

Comparison of VIM-5:**1** and VIM-5:**2** structures with reported MBL-inhibitor complexes, reveals the carboxylate of **2** binds similarly to that of D-/L-captopril (Fig. S10),¹⁷ i.e. via electrostatic interactions with Arg228 in VIM-2 and VIM-5, or equivalent residue, Lys224 in IMP-1 and BcII, which also bind to the β -lactam antibiotic carboxylate (Fig. S11).^{9, 17} Based on these structures we designed and synthesized two compounds (**3** and **4**, Table 1) combining the tryptophan moiety of **2** and the (*S*)-3-mercapto-2-methylpropanal moiety of D-/L-captopril (Supplementary Methods Scheme S1 for **3/4** synthesis), with the aim of identifying broad-spectrum MBL inhibitors.

3 and **4** displayed the most potent inhibition to VIM-2 MBL (Table 1). To investigate their binding modes, we obtained structures of VIM-2 complexed with **3** to 1.20 and 1.16 Å resolution, respectively (Table S3). As predicted, the structures reveal that the thiol of **3** and **4** chelates to and bridges the two zinc ions, as observed for captopril¹⁷ and related thiol based inhibitors^{24, 25} (Fig. 4a–b, S12, S13); as observed for **2**, **3** and **4** are positioned to form electrostatic interactions with Arg228 and Asn233, and hydrophobic interactions with Phe61 and Tyr67 (Fig. 4a–b, S13, S14). **3** and **4**, like **2**, bind in a mode similar to hydrolysed β -lactam product (Fig. S15). Notably, in the VIM-2:**4** structure, the amido group adopts a *cis*-conformation (Fig. 4c). By contrast, the *trans*-conformation is observed in the VIM-2:**3**

structure, where the amido group of **3** binds very similarly to that of D-captopril (Fig. S16). These differences may, in part, explain why **3** is more potent against VIM-2 than **4**; superimposing structures of VIM-2:**3** and VIM-2:**4** with VIM-5:**2** reveals that these compounds bind *via* a similar binding mode (Fig. S14).

3 and **4** manifest broad-spectrum inhibition against the class B MBLs (clinically relevant subclass B1 enzymes VIM-2, VIM-1, VIM-5, IMP-1, NDM-1, SPM-1, and the ‘model MBL’ BcII, the subclass B2 enzyme CphA, and the subclass B3 enzyme L1^{21, 26, 27}), and are more potent than D- or L-captopril (Table 1); **3**, with nanomolar potency for subclass B1 MBLs (IMP-1, VIM-2, VIM-1, VIM-5 and SPM-1), is relatively more potent than **4**. As **1** and **2** occupy slightly different regions of the VIM-5 active site (Fig 2b, 2c, and S17), we synthesized **5** that merges the indole moiety of **1** and the isoquinoline of **2** *via* a linker (Supplementary Methods Scheme S2). However, **5** only showed weak inhibition against VIM-5 (IC₅₀, 41 μM). Comparison of the binding of **3** and **4** to VIM-2 revealed that of **4** is fully reversible, but that, under the tested assay conditions **3** slowly dissociates (Fig. S18).

Since derivatives of **3** and **4** may have clinical prospects due to their synthesis tractability and potent inhibition against subclass B1 MBLs, we tested them against three clinically important multidrug-resistant MBL-producing species: *Escherichia coli*, *Enterobacter aerogenes* and *Klebsiella pneumoniae*. The minimum inhibitory concentrations (MICs) of meropenem against the bacterial strains were determined with and without MBL inhibitors. **3** and **4** potentiated the efficacy of meropenem against VIM-1 and IMP-1-producing strains, with **3** conferring clinically-relevant meropenem susceptibility (MIC < 8 mg/L) in all cases when used at 50 μM (Table 2).

In conclusion, crystallographic analyses of the clinically relevant VIM-5 B1 MBL in complex with enantiomeric isoquinolines **1** and **2** reveal that although they interact with slightly different regions of the VIM-5 active site (Fig. 2), binding of each involves residues on the catalytically important L3 and L10 loops.^{14, 28} The combined crystallographic and solution results also reveal an MBL inhibition mode not involving Zn(II) ion chelation. Comparison of VIM-5:**1** and VIM-5:**2** structures with recently reported inhibitor-MBL structures led to the design of highly potent, broad-spectrum MBL inhibitors **3** and **4**. **3** and **4** were also found to potentiate the efficacy of meropenem against MBL-producing strains. Since **3** and **4** can be readily prepared, they are good starting points for further optimisation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
Outline mechanisms for MBL catalysed β -lactam hydrolysis. Note, in the case of MBLs.
Variations of this mechanism are possible dependent on specific enzyme-substrate combinations.

Fig. 2. Binding of inhibitors **1** and **2** to VIM-5. (a) Chemical structures of (*R*)-enantiomer (**1**) and (*S*)-enantiomer (**2**). (b) View of the active site from the VIM-5:1 (PDB ID: 5N58) (c) and VIM-5:2 (PDB ID: 5N55) complexes. Note, in the VIM-5:2 structure, Cys221 is oxidised to a sulphinic acid resulting in loss of one Zn(II) ion.

Fig. 3. Binding studies of **1** (a), **2** (b), and thiomandelic acid (c) to *apo*-VIM-5 using CPMG NMR. **1** and **2** bind strongly to *apo*- and di-Zn(II)-VIM-5 as indicated by signal density reduction in the presence of *apo*-VIM-5, while thiomandelic acid only binds very weakly to *apo*-VIM-5, indicating that the active site Zn(II) ions are not essential for binding of **1/2**. Assay mixtures contained 50 μ M *apo*-VIM-5 and 50 μ M of the compound of interest, buffered with 50 mM Tris-D₁₁, pH 7.5, and 0.02 % NaN₃ in 90 % H₂O and 10 % D₂O.

Fig. 4. Crystallographic analyses reveal **3** and **4** bind to the VIM-2 MBL via similar modes. (a) View from a structure of **3** complexed with VIM-2 (PDB ID: 5N4S). (b) View from a structure of **4** complexed with VIM-2 (PDB ID: 5N4T). (c) Superimposition of VIM-2:**3** and VIM-2:**4** reveals **3** and **4** bind in similar modes involving zinc chelation. Note that unlike **3**, **4** is bound with *cis*-amide conformation.

Table 1
IC₅₀ values (μM) of **3**, **4**, D-captopril and L-captopril against clinically relevant MBLs

ID	Chemical Structure	Class B1					B2			B3	
		VIM5	VIM2	VIM1	NDM-1	SPM-1	BeII	IMP-1	CphA	L1	
3		0.175	0.055	0.526	24.81	0.890	1.63	0.041	~400	16.21	
4		2.187	1.06	33.73	64.8	0.260	30.4	3.98	>400	97.3	
D-captopril		14.9	4.54	7.452	62.1	1.156	12.03	6.75	>400	67.08	
L-captopril		10.5	2.47	42.84	196.8	1.654	47.82	2.50	>400	73.73	

Table 2
MICs of meropenem (MEM) with and without **3** and **4** against MBL-producing bacteria.

Strain	MEM MIC (mg/liter)						
	DMSO	3 (10μM)	3 (25μM)	3 (50μM)	4 (10μM)	4 (25μM)	4 (50μM)
<i>E. coli</i> + IMP-1	32	32	8	0.5	32	32	32
<i>Ent. aerogenes</i> + IMP-1	32	16	0.5	0.5	32	16	8
<i>K. pneumoniae</i> + IMP-1	32	8	1	0.125	32	8	8
<i>E. coli</i> + VIM-1	16	8	4	0.25	16	16	8
<i>Ent. aerogenes</i> + VIM-1	16	8	8	0.5	16	16	16
<i>K. pneumoniae</i> + VIM-1	16	16	4	0.5	16	16	16