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The identification of new metallo-β-lactamase inhibitor leads from fragment-based screening

Peter Vella [a], Waleed M. Hussein [a], Eleanor W. W. Leung [a], Daniel Clayton [a], David L. Ollis [b], Nataša Mitić [c], Gerhard Schenk [a,c,*], Ross P. McGeary [a,d,*]

[a] The University of Queensland, School of Chemistry and Molecular Biosciences, Brisbane, QLD 4072, Australia
[b] The Australian National University Research School of Chemistry, Canberra, ACT 0200, Australia
[c] National University of Ireland-Maynooth, Department of Chemistry, Maynooth, Co. Kildare, Ireland
[d] The University of Queensland, School of Pharmacy, Brisbane, QLD 4072, Australia

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ABSTRACT

The emergence of metallo-β-lactamases (MBLs) capable of hydrolysing a broad spectrum of β-lactam antibiotics is particularly concerning for the future treatment of bacterial infections. This work describes the discovery of lead compounds for the development of new inhibitors using a competitive colorimetric assay based on the chromogenic cephalosporin CENTA, and a 500 compound Maybridge™ library suitable for fragment-based screening. The interactions between identified inhibitory fragments and the active site of the MBL from *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* were probed by in silico docking studies.

β-Lactam-based antibiotics are the gold standard for treating bacterial infections. However, bacteria have now developed resistance to these drugs through mechanisms such as changes in membrane permeability, efflux pumps and the expression of lactamase enzymes specialising in the destruction of β-lactam antibiotics through hydrolysis.[1,2] Of particular concern is the emergence of the metallo-β-lactamases (MBLs), which are a family of zinc containing metalloenzymes capable of hydrolysing a very broad range of common β-lactam antibiotics, including penams (e.g., benzylpenicillin), cephalosporins (e.g., cephalothin, cefoxitin) and carbapenems (e.g., meropenem and imipenem).[3] Examples of MBLs include VIM-2 and IMP-1, both of which have been isolated following outbreaks of bacterial resistance in hospitals, and both of which now have many documented isoforms.[4–9] MBLs are not inhibited by clavulanic acid, a drug commonly co-administered with β-lactam antibiotics as an inhibitor of other types of lactamases.[10]

The main characteristics of MBLs are the presence of two Zn(II) ions in the active site (although in some cases only one may be needed for catalytic activity) and an overall αββα protein fold (Fig. S1 in Supplementary data).[11–14] The metal ions are proposed to assist in the binding of the antibiotic substrate and the generation of an attacking nucleophile.[14,15] MBLs are classified as B1, B2 or B3 type depending on the residues ligating the metal ions in the active site (Figs. S1 and S2 in Supplementary data). A standard numbering scheme for residues in MBLs has been developed based on sequence alignments for B1, B2 and B3 MBLs, and this scheme is used throughout this Letter.[16] Various catalytic mechanisms have been proposed for MBLs, and were summarised in a recent review.[15] Although the proposed mechanistic strategies vary with respect to the role of the metal ions, the number of metal ions in the active site, the mode of substrate binding and the identity of the rate-limiting step, it is generally accepted that a nucleophilic attack by a metal ion-bound hydroxide onto the antibiotic's four-membered lactam ring triggers ring opening and the hydrolysis of the antibiotic to a therapeutically inactive form.

The imipenemase-1 (IMP-1) MBL from *Pseudomonas aeruginosa* and its isoforms are documented in many outbreaks of antibiotic resistant bacteria in medical facilities worldwide.[17] Examples of serious infections involving *P. aeruginosa* include bacteriaemia, pneumonia, urosepsis and wound infections.[18] Although it is capable of hydrolysing penams (e.g., penicillin), IMP-1 is particularly efficient at hydrolysing cephalosporins and carbapenems.[19] Mobile genetic elements such as plasmids encoding MBLs allow resistance to spread between unrelated bacterial species. It is therefore not

* Corresponding authors. Tel.: +61 7 3365 4144; fax: +61 7 3365 4273 (G.S.); tel.: +61 7 3365 3955; fax: +61 7 3346 3249 (R.P.M.).
E-mail addresses: schenk@uq.edu.au (G. Schenk), r.mcgeary@uq.edu.au (R.P. McGeary).

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surprising that IMP-1 is also found in other bacterial species such as *Klebsiella pneumoniae*.[4,20] The ability for MBLs such as IMP-1 to bestow broad-spectrum antibiotic resistance to bacterial pathogens, transfer resistance genes between bacterial species, the lack of a clinically useful inhibitor and the diminishing number of treatment options available for use against resistant bacterial infections highlights the need for the development of potent inhibitors against such MBLs.[21,22]

Fragment-based screening has proved to be an effective strategy for the discovery and development of drug leads.[23–25] In this approach, low molecular weight ligands that bind to proteins of interest with weak binding affinities (typically around 1 mM) are identified. NMR spectroscopy or X-ray crystallography are subsequently used to determine the location and three-dimensional binding modes of these fragments. Both competitive and non-competitive binders can be used as starting points for further drug lead development, by tethering fragments together to develop more potent ligands, and by designing drug-like properties into these compounds.[26] Successes using this approach include the development of a CDK2 inhibitor now in clinical trials by Astex Therapeutics and the development of a Bcl-2 inhibitor by Abbott Laboratories.[27,28] A fragment based screening approach based around the chromogenic cephalosporin CENTA (see **15**, Scheme 1 in the Supplementary data) and the 500-compound Maybridge™ R03 fragment library was therefore devised here to identify new inhibitors against IMP-1.[29,30] Although this approach was expected to initially yield compounds with inhibition constants only in the low mM to high μM range, the philosophy of the fragment-based approach is to first identify, and then develop such compounds into more potent inhibitors by synthetic modifications guided by in silico docking or crystallography studies.[31,32] The synthesis of CENTA, assays used, the in silico docking approach and other experimental procedures employed in this study are detailed in the Supplementary data.

The aim of the present study was to identify new inhibitors of MBLs using a fragment-based screening approach. As a target we selected IMP-1 from *P. aeruginosa* and *K. pneumoniae* as these pathogens have already displayed significant resistance to many, if not most, commonly used antibiotics. To search for new inhibitors of IMP-1, the 500 compound Maybridge™ R03 fragment library was screened. A 96-well plate assay was used to facilitate rapid throughput in the analysis. Captopril, a known inhibitor of MBLs was used to test the sensitivity of this assay.[33] The D- and L-diastereomers of captopril differ in the chirality of the proline moiety and are both known to inhibit MBL activity, however L-captopril is commercially available and was thus used for the assays.

The inhibition of IMP-1 by L-captopril was assessed using both a plate reader and a cuvette assay using benzylpenicillin as a substrate (Figs. S3a and S3b in Supplementary data). Interestingly, CENTA was not a suitable substrate since captopril appeared to initiate its hydrolysis (data not shown). The mode of inhibition is competitive with a K_{ic} of 12.5 ± 2.4 μM (cuvette assay) and 7.2 ± 1.2 μM (96-well plate assay) demonstrating that the 96-well high-throughput assay provides reliable kinetic data. In the initial fragment screening assays the percent inhibition was determined by comparing the rate of CENTA turnover between assays where the fragment was present (at 1 mM concentration) or absent. The inhibition cut-off was set at 50%, with fragments meeting this criterion being further analysed using inhibition assays as described for L-captopril (vide supra and Figs. S3a and S3b in the Supplementary data). The remaining fragments were not further assessed.

Ten compounds with inhibition constants within the high micromolar to low millimolar range were thus identified and their inhibition constants, K_i, and mode of inhibition were assessed (compounds **1**–**10**, Fig. 1). The inhibition constants are summarised in Table 1, and a plot of the K_i determination for the most potent compound, fragment **1**, appears in the Supplementary data (Fig. S4). Fragment **1** also has the highest ligand efficiency of the compounds tested.[34] Although the compounds generally exhibited mixed inhibition (both competitive and uncompetitive inhibition modes were observed), the uncompetitive mode of inhibition was usually dominant. Only fragment **10** was observed to inhibit MBL activity in a predominantly competitive way. As the aim of a fragment based screen is to identify small molecules that may form a part of a larger, more potent inhibitor, the discovery of a number of uncompetitively bound fragments is encouraging for the development of lead compounds to inhibit MBLs. The uncompetitive mode of inhibition indicates that the fragment does not prevent the substrate CENTA from binding to the active site; instead, a ternary enzyme–substrate–inhibitor (ESI) complex is formed that does not promote hydrolysis of the substrate. This enzyme–substrate–inhibitor (ESI) may provide the basis for the synthesis of potent and specific IMP-1 inhibitors. To gain insight into its possible structure, in silico docking was employed. The aim was to dock the substrate, CENTA, and a fragment into the crystal structure of IMP-1 (PDB code: 1JJT).[35] The fragments selected for docking were **1** (strongest biding affinity), **6** (an uncompetitive inhibitor fragment) and **10** (a competitive inhibitor fragment) (Ta-

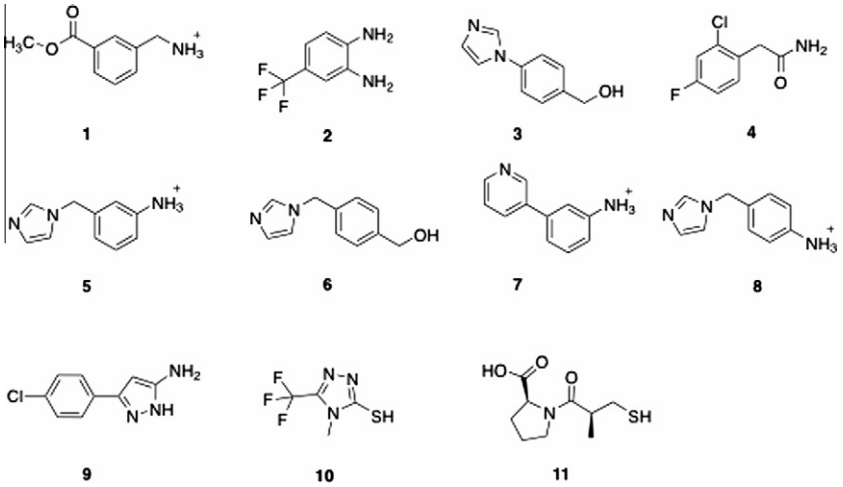

Figure 1. L-Captopril (**11**) and fragments **1**–**10** that were identified from the Maybridge™ library as potential leads to design and synthesise novel MBL inhibitors.

Table 1
Inhibition constants for L-captopril and the ten most potent fragment compounds from the Maybridge™ library

Number	Identifier	K_{iuc} (mM)	K_{ic} (mM)
1	mo07352	0.41 ± 0.10	0.89 ± 1.36
2	btb7340	0.43 ± 0.07	0.99 ± 0.87
3	cc18509	0.50 ± 0.09	–
4	tl01011	0.68 ± 0.17	0.76 ± 0.45
5	cc43214	0.78 ± 0.14	1.64 ± 1.60
6	cc43309	0.79 ± 0.10	–
7	cc39814	0.90 ± 0.17	0.51 ± 0.27
8	cc43314	1.18 ± 0.23	1.51 ± 1.08
9	km01548	1.20 ± 0.30	–
10	sb00671	–	0.97 ± 0.60
11	Captopril	–	0.0125 ± 0.0024[a]

–Indicates result >2 mM.
[a] Substrate used in the assays was penicillin G, cuvette assay.

ble 1). As the residues Glu 60, Val 61 and Trp 64 form part of a flexible loop on IMP-1, the docking was run with these residues marked as flexible. The reliability of the docking method was tested using the competitive inhibitor D-captopril as a probe.

D-Captopril docked into the active site of IMP-1 (Fig. S5) shows a similar binding mode to that observed for crystal structures of MBLs that have D-captopril bound, such as the B1-type MBL BlaB (PDB code: 1M2X)[36] where the sulfur atom of the inhibitor coordinates in a bridging mode to both metal ions in the active site. Since D-captopril occupies the space close to the metal ions it effectively competes with the substrate for a spot in the immediate vicinity of the catalytic centre, a binding mode that is consistent with competitive inhibition. Subsequently, the substrate CENTA and the most potent of the fragment compounds, **1**, were docked into the active site of IMP-1, first separately, then as an ESI complex. Fragment **1** docks into the active site of IMP-1 with two plausible conformations. In the first conformation, the N of the amino group of **1** occupies a position close to both metal ions in the active site (Zn1: 4.0 Å; Zn2: 3.2 Å; Fig. 2a). In the second conformation the fragment interacts through π-stacking with residue Trp 64, which is located on the flexible loop close to the active site of IMP-1 (Fig. 2b). As the inhibition data (Table 1) indicate fragment **1** is a mixed-type inhibitor, we thus assign the first, lower energy conformation as the competitive mode of binding and the second as the uncompetitive one. Consistent with the uncompetitive binding mode of fragment **6**, the docking solution suggests that this fragment does not bind to the zinc ions within the active site (Fig. S6). However, the docking shows fragment **6** interacting through hydrogen bonding with Lys 224 (through the N atom on the imidazole ring) and Ser 119 of the enzyme (through the hydroxyl group). The docking solution for fragment **10** suggests that it closely binds a zinc ion in the active site using two nitrogen atoms from the triazole ring (Zn1: 2.6, 2.5 Å; Zn2: 2.5 Å), consistent with the competitive mode of inhibition observed from the kinetic assays (Fig. S7).

The highest ranked docking solution of the substrate CENTA docked into IMP-1 is shown in Figure 2c. CENTA docks into the active site with the nitrogen of the lactam ring positioned directly in front of the active site metal ions, thus being in a position that facilitates a nucleophilic attack by a metal ion-bound hydroxide. The carbonyl group on the lactam ring of CENTA docks closely to Zn1 and the carboxylic acid group is shown interacting with Lys 224. The observation of this particular interaction through in silico docking is encouraging as previous work by Haruta et al. have demonstrated that substitution of this lysine residue to an arginine, alanine or glutamic acid in IMP-1 results in an increased K_m, and thus weaker substrate binding.[37] Furthermore, they hypothesised that the positive charge of the lysine was important for the binding of the carboxylic acid group of β-lactam antibiotic

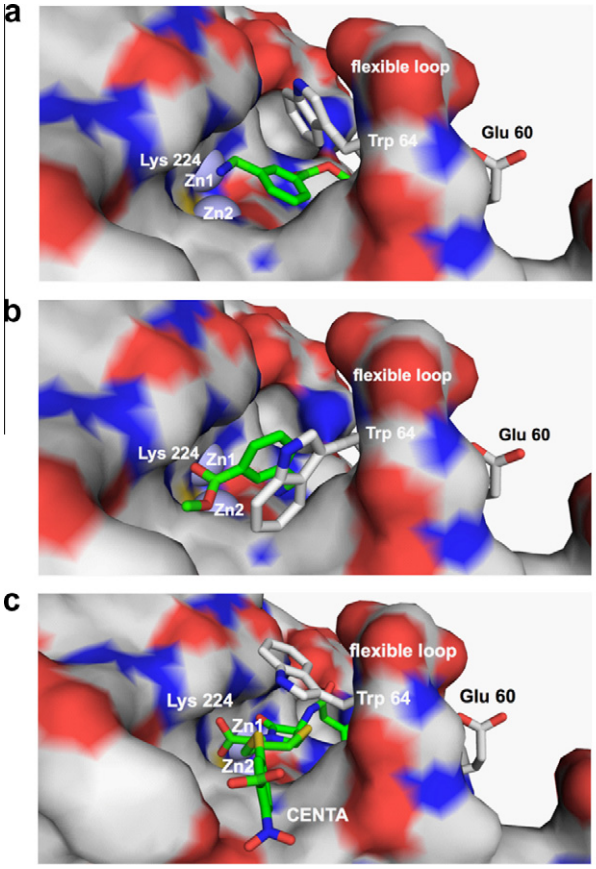

Figure 2. (a) Surface view of the IMP-1 active site for the highest Autodock Vina score conformation of IMP-1 with fragment **1** docked into the active site in a proposed 'competitive mode'. (b) Fragment **1** docked into the active site in a proposed 'un-competitive mode'. (c) CENTA substrate docked into the active site. For clarity, Trp 64 and Glu 60 of the flexible loop adjacent to the active site has been given a 'stick' representation. Atom colours are as follows: blue—nitrogen, red—oxygen, white—carbon (on IMP-1), green—carbon (on inhibitor or CENTA), gold—sulfur, purple—zinc active site metals.

substrates, and that this interaction was one possible explanation for why MBLs generally lack activity towards β-lactam monobactam antibiotics, which lack this particular carboxylic acid group.

Since the ESI complex may point towards the design of more potent next generation inhibitors for MBLs, docking was used to model the ESI complex by docking CENTA into IMP-1 with fragment **1** already bound (Fig. S8 in Supplementary data), and a superposition of CENTA (Fig. 2c) and compound **1** in the uncompetitive binding mode (Fig. 2b) docked into IMP-1 was carried out. While the docking approach does not account for all protein dynamics during substrate and/or inhibitor binding, the superimposed structure does show that fragment **1** and CENTA are in close proximity near the active site, and are thus amenable to linking together to form a putative, more potent competitive inhibitor of IMP-1 (Fig. S9 in Supplementary data). For example, extending functionality from the methyl ester may allow occupation of a pocket that is present to the left of Lys 224, and additional pendant groups could be attached to the phenyl ring to bind the Zn(II) metals within the active site.

In conclusion, a number of compounds with sub-millimolar inhibition constants (both competitive and non-competitive modes of binding) from the Maybridge™ R03 fragment library, have been identified as inhibitors of the IMP-1 MBL. This work also confirms the use of the chromogenic cephalosporin CENTA as a suitable substrate and alternative to nitrocefin for the purpose of

inhibitor screening. The identification of ten inhibitor leads which predominantly show an uncompetitive mode of inhibition provides a platform for the further development of potent compounds against IMP-1 in particular, and MBLs in general.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.04.027.

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