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In silico fragment based design identifies potent β-lactamase inhibitors

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ABSTRACT

Zinc ion dependent β-lactamases (MBLs) catalyse the hydrolysis of almost all β-lactam antibiotics and resist the action of clinically available β-lactamase inhibitors. We report how application of *in silico* fragment-based molecular design employing thiol-mediated metal anchorage leads to potent

MBL inhibitors. The new inhibitors manifest potent inhibition of clinically important B1 subfamily MBLs, including the widespread NDM-1, IMP-1 and VIM-2 MBLs; notably, they also inhibit some clinically relevant Class A and D serine-β-lactamases. The inhibitors show selectivity for bacterial MBL enzymes compared to human MBL fold enzymes (DCLRE1A and DCLRE1B). Co-crystallization of one inhibitor, which shows potentiation of meropenem activity against MBL-expressing *Enterobacteriaceae,* with the VIM-2 MBL reveals a novel binding mechanism, involving interactions with residues from conserved active site bordering loops.

INTRODUCTION

β-Lactam-containing molecules remain the single most important antibacterials.[1-2] β-Lactams inhibit transpeptidases (or penicillin-binding proteins, PBPs) involved in cell wall biosynthesis by reacting with a catalytically crucial nucleophilic serine reside. Bacteria have developed resistance to β-lactams most importantly by β-lactamase catalysed hydrolysis. There are four classes of β-lactamases:[3] classes A, C, and D are serine-β-lactamases (SBLs), mechanistically related to the PBPs; class B are metallo-β-lactamases (MBLs), employing zinc ions in catalysis.

Co-administration of a β-lactam antibacterial with class A β-lactamase inhibitors (clavulanic acid,[4] tazobactam,[5] sulbactam) has successfully overcome some SBL-mediated resistance. Avibactam,[6] an inhibitor of class A, C, and some class D SBLs, has recently been introduced into the clinic. However, as yet there are no reports of clinically useful class B MBL inhibitors. MBLs are a concern because they catalyze hydrolysis of almost all β-lactam antibacterials, including the carbapenems, which are commonly used for treatment of severe and highly resistant infections. MBLs are divided into the B1, B2, and B3 subclasses based on metal site occupancy and sequence similarity.[7-9] Subclass B1 MBLs, which employ two zinc ions (Zn1/Zn2) are the most clinically

relevant and include the New Delhi metallo-β-lactamase (NDM-1), Verona integron-encoded MBL (VIM), and Imipenemase (IMP) types of B1 MBLs.

Combating increasing resistance mediated by MBLs requires identification of either novel antibacterials, MBL resistant β-lactams, (both substantial undertakings), or developing an MBL inhibitor for co-administration with an existing β-lactam. Here we report how the application of *in silico* fragment-based[10-11] molecular design led to identification of potent inhibitors of clinically relevant MBLs and certain SBLs. The *in silico* studies employed a structure-guided approach in which fragments were anchored to the di zinc ion centre *via* a thiol and then optimised for binding *via* hydrophilic and electrostatic interactions. Whilst such metal anchorage (or 'support ligand') based approach has been applied in dynamic combinatorial based identification[12] of ligands to MBLs, to our knowledge this is the first example of the application of an *in silico* fragment-based approach to the production of specific MBL inhibitors.

RESULTS AND DISCUSSION

De Novo Ligand Design

The *de novo* molecular design program SPROUT generates potential ligands *via* docking of fragments into targeted regions; subsequent user-directed assembly using template spacers to link these fragments together yields synthetically tractable scaffolds (Figure 1a). Analysis of an NDM-1 crystal structure complexed with hydrolysed ampicillin (PDB code 3Q6X)[9] using SPROUT[11] led to the identification of specific regions within the active site for targeting with *in silico*-generated fragments, i.e. regions proximal to Lys224, the Zn2 metal ion, the nucleophilic hydroxide that "bridges" the two zinc ions, and the conserved Trp87 that makes hydrophobic interactions with

the aromatic ampicillin C6 side chain (Figure 1b). Importantly, while details differ between individual MBLs, these features are predicted to be crucial for β-lactam binding by most, if not all, B1 MBLs.[13] This design approach led to the identification of simple benzyl thiol **1** (Fig. 2) as a potential NDM-1 substrate-competitive inhibitor. The predicted interactions of **1** with NDM-1 include the carboxylate with the Lys224 side chain and Zn2 ion; the anchoring thiol with both Zn1 and Zn2 atoms; and the aryl ring with Trp87 via π-stacking interactions (Figure 2a). Compound **1** was synthesized using a Suzuki coupling (67%)[14] between 5-bromophthalide and phenyl boronic acid to give a lactone which was ring-opened using Me_3SiI to give the corresponding iodide (62%). Iodide displacement using thiourea, followed by hydrolysis gave **1** (81%; see Methods and Supplementary Information).

Using a fluorogenic MBL assay[15] **1** was evaluated for inhibition of recombinant NDM-1, IMP-1 and VIM-2. The results (Table 1) reveal **1** to be an inhibitor with sub-micromolar IC_{50} values for all three of the tested B1 MBLs, with IC_{50} values 232 – 234 nM; These values compare favourably with the thiol-MBL inhibitors such as *L*-captopril for which IC_{50} values against B1 MBLs are in the micromolar range.[13]

Crystallographic studies

To investigate the binding of this class of MBL inhibitors, a structure of **1** with VIM-2 was obtained using co-crystallization (1.74 Å resolution; space group $C2_1$; see Supplementary Information Table 1). As predicted by the design studies, analysis of the crystal structure confirms that the thiol of **1** acts as a metal binding ligand that displaces the proposed 'hydrolytic' water molecule (or hydroxide) that bridges the two active site metal ions (Figure 2b). The crystallographically observed conformation of the biphenyl unit was not, however, as predicted by design (Figure 2a). Somewhat unexpectedly the carboxylate of **1** is also positioned to interact with

Trp87 and Asp117 *via* contact to water molecules, rather than with Arg228. In the crystal structure, **1** presents two binding faces as observed with the predicted substrate or observed product binding modes.[13] One face is hydrophobic and is involves interactions with the substantially hydrophobic L3 loop via a π-stacking interaction of the aryl ring of **1** with Tyr67 and a T-shaped stacking between **1** and Trp87. The other binding face involves electrostatic and hydrogen bonding interactions between donor residues from L10 loop, a cation–π interaction between Arg228 and the phenyl ring, and hydrogen bonding of the carbonyl oxygen and the amide bond of Asn233.

Structure Activity Relationship study

Following the encouraging levels of inhibition observed with **1**, we conducted structure activity relationship (SAR) studies. Modification of the phenyl ring of **1** (Table 1) revealed no clear trend in potency when changing the electron density or steric bulk via incorporation of bromine (**17**) or five-membered ring substituents (**15** and **16**), implying that the π-stacking interaction with Tyr67 is not crucial for high affinity. Similarly, positioning substituents in the *ortho*-position of the phenyl ring (**4, 9** and **10**), to investigate whether changes to the dihedral angle observed in the complex with VIM-2 might affect affinity, did not yield significant potency changes. The importance of the spatial arrangement of functional groups was then investigated via compound **19** (see Supplementary Information Fig. 4) which has the thiol directly on the aromatic ring and has a spacer to the carboxylic acid. The thiolactone **18,** (see Supplementary Information Fig. 3) was synthesised to test whether it could act as a pro-drug, potentially undergoing hydrolysis to **1** (see Supplementary Information Table. 2). Neither **18** nor **19** manifested significant activity against VIM-2 (IC_{50} > 100 μM) showing that the spatial arrangement of the functional groups is

important and that the thiolactone does not appear able to act as a masked form of the thiol and carboxylate moieties, at least against VIM-2.

Molecules **2**-**17** were evaluated for inhibition against the clinically relevant subclass B1 enzymes NDM-1 and IMP-1 (Table 1 and Supplementary Information Table 2) and selected compounds (**4**-**8**) against the model MBL BcII and the hybrid B1/B2 like MBL SPM-1.[16] The results reveal **4-8** to be active against BcII and SPM-1 (with IC_{50} values 0.78 – 7.8 μM, see Supplementary Information Table. 3. Moreover, except for **11**, all compounds manifested sub-micromolar IC_{50} values against IMP-1 and several compounds showed also sub-micromolar activity against NDM-1 (**5**, **6**, **7**, **8** and **13**).

We used ^{1}H CPMG NMR to investigate binding of **1** and **9** to VIM-2, and ^{19}F NMR for binding to NDM-1 (see Supplementary Information Fig. 6). In agreement with the inhibition data, **1** and **9** bind with K_D values < 1 μM to VIM-2 and **9** out competes **11** for binding site to NDM-1 (see Supplementary Information Fig. 7).

Selectivity screening

Clinically relevant MBL inhibitors should show selectivity towards the bacterial MBLs over human metallo-enzymes, including human MBL-fold enzymes.[17] We tested **1**, **5**, **13** and **17** against the human MBL fold enzymes, DNA cross-link repair enzymes 1A and B (DCLRE1A and DCLRE1B). DCLRE1A was only weakly inhibited by **1** (IC_{50} 247 ± 154 μM) and DCLRE1B was inhibited by **1**, **5** and **17** (IC_{50}s 149 ± 27, 268 ±112 and 185 ± 50 μM, respectively), revealing some selectivity of the compounds towards the bacterial MBLs. Compound **1** showed good aqueous solubility and underwent moderate metabolism in human liver microsomes (see Supplementary Information).

We also screened **1**, **7**, **8** and **9** against TEM-1, AmpC, and OXA-10, representative Class A, C, and D SBLs, respectively. Interestingly, **8** and **9** inhibited TEM-1 and **9** inhibited OXA-10 (IC_{50}s 32.8, 5.5 and 37.3 μM, respectively) (see Supplementary Information Table. 4). These results suggest these inhibitors may interact with SBLs and MBLs via related binding modes, which would be consistent with our proposal that the compounds act as β-lactam mimics, though we have not been able to obtain structures of these compounds in complex with SBLs.

Antibacterial Activity

The more potent inhibitors were then screened in antimicrobial assays, studying the effect of co-administration on the minimal inhibitory concentration (MIC) of the clinically important carbapenem meropenem in MBL producing strains of *E. coli* and *K. pneumoniae* (species where NDM-1 production is a growing clinical problem).[18] Tests in the absence of inhibitors showed meropenem MICs against MBL-producing bacteria of >128 μg/ml, and that **1** alone has no antibacterial activity (Table 2). Encouragingly, at 100 μg/ml all tested compounds, apart from **11**, reduced the MIC of meropenem against NDM-1-producing strains. Compound **16** showed the strongest activity, reducing the meropenem MIC to 8 μg/ml against *K. pneumoniae.*

CONCLUSIONS

Overall the results reveal how computer aided design can generate a novel class of β-lactamase inhibitors that are active against clinically important MBLs, and that can reduce MICs of meropenem against MBL-producing bacteria. Unlike some reported MBL inhibitors,[19] the new type of MBL inhibitor described here does not function by removing the zinc ions from the active site as shown by NMR studies. While utilizing the free thiol group to bind the MBL active site by

intercalating between the two zinc ions has precedent,[13] the crystallographically observed mode of binding for the appended fragment has not been previously observed. The observation of activity for the designed compounds against several different types of clinically relevant MBLs is notable because inhibition of a spectrum of MBLs is a likely prerequisite for clinical application of an MBL inhibitor. Further, multiple variants of the B1 MBLs are being identified.[7] Although the compounds described here are unlikely to be of direct clinical utility, the work suggests that computational chemistry can have an important role in designing inhibitors that are active against both current and future predicted antibacterial resistance mediated by β-lactamases.

EXPERIMENTAL SECTION

The Supporting Information contains a complete general experimental section, including all procedures and equipment used. Chemicals were purchased from commonly used suppliers (Aldrich, Acros, and Alfa Aesar) and were used without further purification.

General procedure for the Suzuki cross coupling reaction

The boronic acid (1.1 eq) was added to a stirred solution of 5-bromophthalide (1.0 eq), potassium carbonate (1.0 eq) and tetrakis(triphenylphosphine) palladium (0) (0.05 eq) in THF (4 mL) and water (2 mL). The reaction mixture was heated to reflux for 12 h. The reaction mixture was filtered through Celite® then concentrated *in vacuo*. The residue was diluted with water (20 mL) and extracted using dichloromethane (3 × 20 mL). The combined organic extracts were dried ($MgSO_4$) and concentrated *in vacuo* to give a pale yellow solid which was purified using flash column chromatography to afford the coupled products.

General procedure for lactone opening with iodotrimethylsilane forming the iodide species

The desired benzofuranone (1.0 eq) was dissolved in dichloromethane (4.5 mL). TMSI (1.5 eq) was added and the reaction was refluxed under nitrogen for 3 h. After this time, the mixture was cooled to room temperature and quenched with water (3 mL). The precipitate was isolated using filtration and washed with water (2 × 10 mL) to give the desired product as an off-white solid which was used without further purification.

General procedure for the introduction of the thiol functionality[20]

A mixture of thiourea (1.1 eq) in THF (3 mL) was heated to reflux. To this mixture was added the iodine-containing open lactone product (1.0 eq). The reaction mixture was heated to reflux for 16 h under an atmosphere of nitrogen and the reaction mixture was cooled to room temperature. The mixture was then concentrated *in vacuo*. The residue was re-suspended in 2M aqueous NaOH (5 mL) and the solution refluxed for 3 h. The reaction mixture then acidified with 2M aqueous HCl to ~pH2. The colourless precipitate was isolated by filtration.

ASSOCIATED CONTENT

Supporting Information.

The following files are available free of charge.

Experimental procedures, characterization of intermediates and target compounds, description of protein production and purification, biological assays and determination of IC50 values. (PDF)

Accession Codes

PDB code for VIM-2 with bound **1** is 5K48

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Author Contributions

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Notes

The authors declare no competing financial interests.

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ABBREVIATIONS USED

MBL, metallo-β-lactamase; SBL, serine-β-lactamase; TMSI, iodotrimethylsilane.

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supplementary information (ESI) available: Procedures for protein expression and purification, 19F-labelling, crystallisation, data collection, and structure determination, table of crystallographic data, table of crystallographic parameters and refinement statistics, figures showing binding mode and distances, procedures for mass spectrometry measurements, differential scanning fluorimetry measurements, stopped-flow measurements and other kinetics measurements. See DOI: 10.1039/c4sc01752h Click here for additional data file. *Chem Sci* **2015,** *6* (2), 956-963.

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Figure 1. Design of broad spectrum MBL inhibitors. A) Key elements used in the SPROUT computational design process leading to the identification of the putative broad spectrum MBL inhibitors. B) Outline mechanism for SBLs and MBLs.

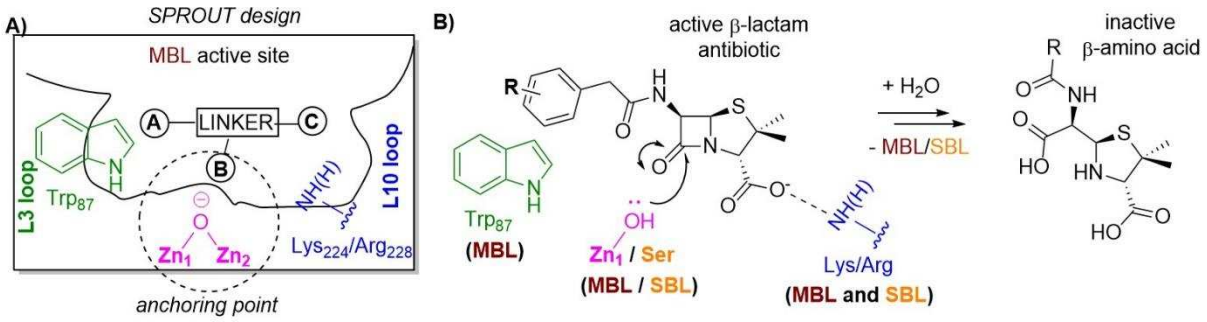


Figure 2. Crystallographic analysis of predicted binding mode for designed MBL inhibitors. (A), (C) and (D) 1 docked into the active site of NDM-1, TEM-1 and OXA-10, respectively, and (B) View from a crystal structure of compound 2 co-crystalized with VIM-2 (PDB ID: 5K48).

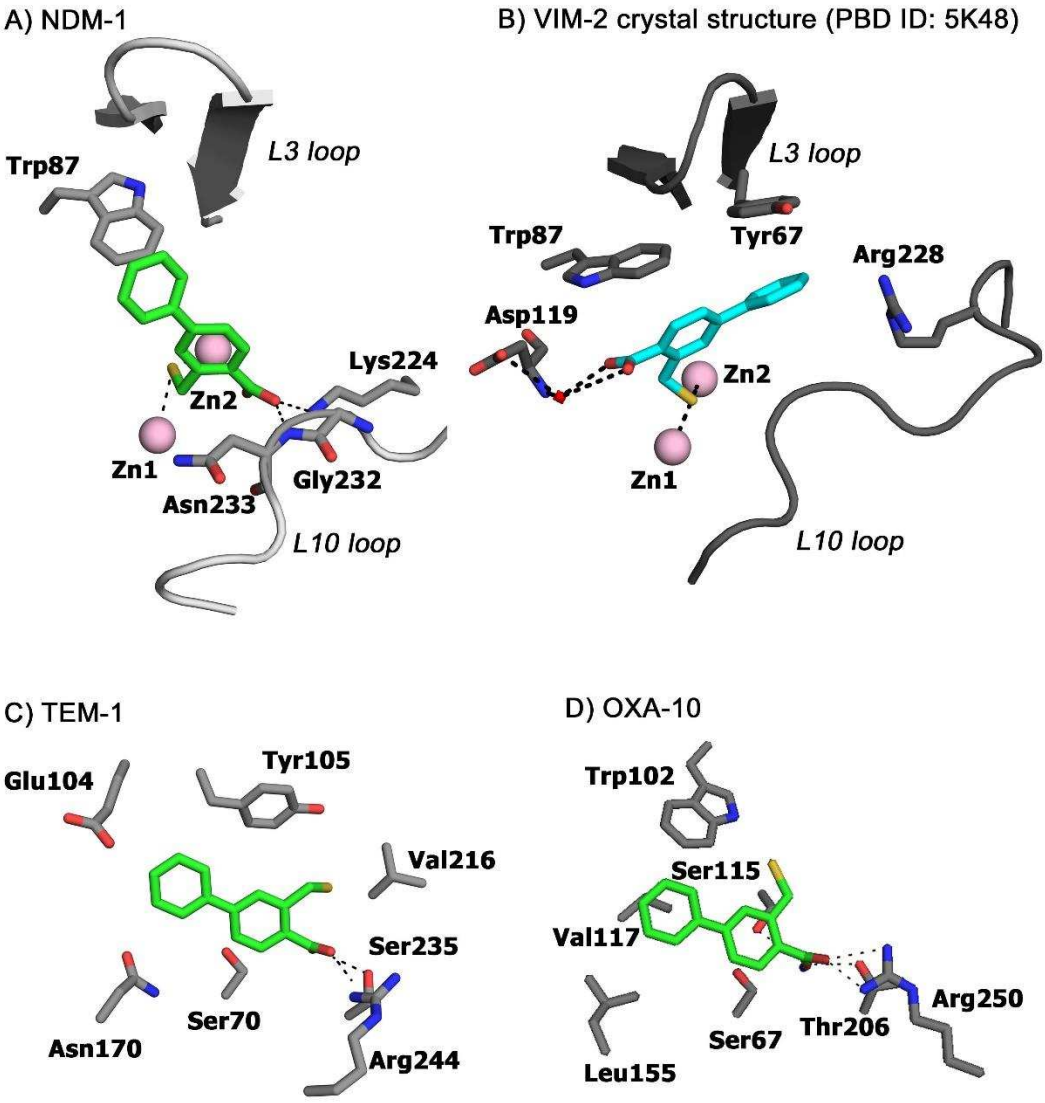
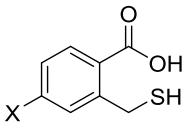


Table 1. IC_{50}s[a] of *de novo* design thiol series against clinically relevant MBLs.



No.	X	cLogP[a]	IC_{50} NDM-1 (μM)	IC_{50} VIM-2 (μM)	IC_{50} IMP-1 (μM)
1	Ph	3.76	5.59 ± 0.052	0.234 ± 0.044	0.232 ± 0.043
4	2-ClC_6H_4	4.37	2.85 ± 0.037	0.180 ± 0.098	0.309 ± 0.067
5	3-ClC_6H_4	4.37	0.311 ± 0.059	0.072 ± 0.059	0.147 ± 0.057
6	4-ClC_6H_4	4.37	0.423 ± 0.027	0.109 ± 0.039	0.069 ± 0.052
7	3-FC_6H_4	3.90	0.597 ± 0.028	0.207 ± 0.069	0.397 ± 0.089
8	4-FC_6H_4	3.90	0.711 ± 0.017	0.231 ± 0.030	0.307 ± 0.094
9	2,3-ClC_6H_4	4.97	1.16 ± 0.057	0.045 ± 0.031	0.378 ± 0.062
10	2-$CF_3C_6H_4$	4.64	8.82 ± 0.052	0.158 ± 0.089	0.480 ± 0.045
11	3,5-$CF_3C_6H_4$	5.52	43.95 ± 0.063	0.459 ± 0.073	1.723 ± 0.047
12	2-Naphthyl	4.75	2.42 ±0.032	0.071 ± 0.031	0.220 ± 0.035
13	4-EtC_6H_4	4.72	0.577 ± 0.029	0.107 ± 0.038	0.057 ± 0.037
14	3-$OEtC_6H_4$	3.96	7.59 ± 0.037	0.184 ± 0.165	0.323 ± 0.037
15	2-Furyl	2.82	10.21 ± 0.040	0.365 ± 0.023	0.393 ± 0.035
16	3-Thienyl	3.54	6.78 ± 0.091	0.174 ± 0.127	0.189 ± 0.049
17	Br	2.88	> 100	0.170 ± 0.018	0.804 ± 0.030

[a]cLogP calculated using Marvin Sketch (ChemAxon)

[b] The IC_{50} values are shown as the mean ± SD from minimum three separate experiments

Table 1. Determination of Meropenem MIC against bacterial strains when co-administered with the designed β-lactamase inhibitors.

Compound Number	**X**	**Meropenem MIC (μg/mL) NDM-1 producing *K. pneumoniae* (ATCC 5055)**	**Meropenem MIC (μg/mL) NDM-1 producing *E. coli* (MG1655)**
Meropenem		> 128	> 128
1	Ph	32	16
17	Br	64	32
11	3,5-CF_3 Ph	>128	>128
12	2-Naphthyl	32	32
13	4-Et Ph	64	32
14	3-OEt Ph	16	64
15	2-Furyl	64	32
16	3-Thienyl	8	16

Table of Contents Graphic