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Studies on the Inhibition of AmpC and other β-Lactamases by Cyclic Boronates

Samuel T. Cahill[a], Jonathan Tyrrell[e], Iva Hopkins Navratilova[c], Karina Calvopiña[d], Michael A. McDonough[a], Ricky Cain[b], Colin W. G. Fishwick[b], Matthew B. Avison[d], Timothy R. Walsh[e], Christopher J. Schofield[a*], and Jürgen Brem[a*]

[a]Department of Chemistry, University of Oxford, Chemistry Research Laboratory, 12 Mansfield Road, Oxford, OX1 3TA, United Kingdom.
[b]School of Chemistry, University of Leeds, Leeds LS2 9JT, United Kingdom.
[c]University of Dundee, Dow Street, Dundee, DD1 5EH, UK; Research Complex at Harwell, Rutherford Appleton Lab, Harwell, Oxford, OX11 0FA, United Kingdom.
[d]School of Cellular and Molecular Medicine, University of Bristol, Biomedical Sciences Building, Bristol BS8 1TD, United Kingdom.
[e]Department of Medical Microbiology & Infectious Disease, Institute of Infection & Immunity, UHW Main Building, Heath Park, Cardiff, CF14 4XN, United Kingdom.

*Addresses for correspondence: Dr. Jürgen Brem; Email: jurgen.brem@chem.ox.ac.uk or Prof. Christopher Schofield; Email: christopher.schofield@chem.ox.ac.uk.

Abstract

The β-Lactam antibiotics represent the most successful dug class for treatment of bacterial infections. Resistance to them, importantly via production of β-lactamases, which collectively are able to hydrolyse all classes of β-lactams, threatens their continued widespread use. Bicyclic boronates show potential as broad spectrum inhibitors of the mechanistically distinct serine- and metallo- serine (SBL) and metallo (MBL) β-lactamase families. Together with reported studies on the structural basis of bicyclic boronate inhibition of class A, B and D β-lactamases, biophysical studies including crystallographic analysis of a bicyclic boronate in complex with the clinically important class C AmpC SBL from Pseudomonas aeruginosa supports the proposal bicyclic boronates mimic tetrahedral intermediates common to SBL and MBL catalysis. Microbiological studies on the clinical coverage (in combination with meropenem) and induction of β-lactamases by bicyclic boronates further support the promise of such compounds as broad spectrum β-lactamase inhibitors.

Keywords: β-Lactamase inhibition, β-lactam antibiotic resistance, cyclic boronate inhibitor, metallo and serine β-lactamases, transition state analogue, surface plasmon resonance, β-lactamase induction, clinical coverage, carabapenem.

Introduction

The β-Lactam antibiotics represent the most successful dug class for treatment of bacterial infections[1]. Resistance mechanisms, particularly the production of β-lactamases, which collectively are able to hydrolyse all the classes of β-lactam antibiotic, endanger their continued widespread use[2] (Figure 1A). Success has been had in the treatment of bacterial infections exhibiting resistance by some serine-β-lactamases (SBLs), particularly Ambler class A enzymes, via co-adminstration of a penicillin with a β-lactam based SBL inhibitor, i.e. clavulanic acid[3-4], sulbactam,[5] or tazobactam[6]. The recent introduction of avibactam, which is active against class A, C, and some class D β-lactamases, demonstrates the viability of non β-lactam based β-lactamase inhibition and is an important step in more broadly combating SBLs[7]. Acyclic boronic acids have long been known to inhibit nucleophilic enzymes, including SBLs[8-9] (Figure 1B). In co-administration with meropenem the (predominantly) monocyclic boronic acid, vaborbactam (Figure 1C), has been introduced for treatment of complicated urinary tract infections (cUTI)[10]. Vaborbactam is relatively potent in inhibiting class A SBLs, including the KPC carbapenemases, but is not active against MBLs and, at least, some clinically relevant SBLs[10].

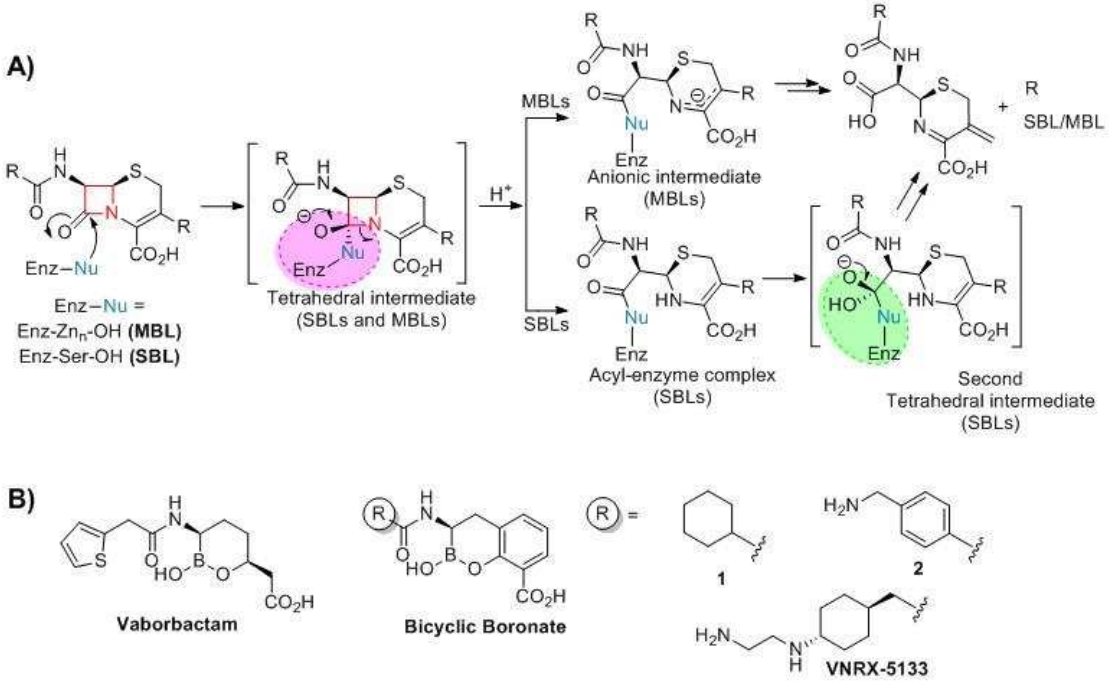

Figure 1. A) Outline mechanisms of serine- and metallo-β-lactamase (SBL and MBL) catalysis, exemplified with a cephalosporin. Note products can be produced in different

tautomeric forms. The tetrahedral intermediate, common to both SBLs and MBLs, may be mimicked by cyclic boronates. **B)** Structures of Vaborbactam and bicyclic boronate β-lactamase inhibitors VNRX-5133, **1** and **2**.

By contrast with the SBLs, to date there are no clinically useful inhibitors of the metallo-β-lactamases (MBLs, Ambler class B)[11], which are structurally and mechanistically distinct from the SBLs and which are structurally heterogeneous (B1-3 MBL subfamilies)[12] (Figure 1A). The ability of the MBLs to hydrolyse β-lactam based SBL inhibitors prohibits their use against bacteria producing both MBLs and SBLs[12]. The observation that MBLs can bind and hydrolyse avibactam, albeit slowly[13], as well as SBL-mediated resistance to avibactam suggests that the future use of avibactam will be jeopardised by β-lactamases[1314]. Thus the development of dual-action SBL and MBL β-lactamase inhibitors is of interest.

We have reported that boronates with a (at least predominantly, in solution) bicyclic scaffold are able to inhibit representatives of all four Ambler classes[15-17]. These inhibitors are proposed to mimic the tetrahedral intermediates in β-lactam hydrolysis common to both SBLs and MBL[15-16]. We now report a crystal structure of a bicyclic boronate in complex with the clinically important class C AmpC β-lactamase from Pseudomonas aeruginosa. Together with reported studies on the structural basis of bicyclic boronate inhibition of class A, B and D β-lactamases and other biophysical analyses our results support the proposal that bicyclic boronates mimic the tetrahedral intermediates common to both serine- and metallo-β-lactamase hydrolysis. Microbiological studies on the clinical coverage (in combination with meropenem) and induction of β-lactamases by bicyclic boronates validate the potential of such compounds as broad spectrum β-lactamase inhibitors.

Experimental Procedures

Enzyme production

Recombinant VIM-2, with an N-terminal His-tag, was produced using the reported pOPINF construct[18] in E. coli BL21(DE3) pLysS cells using 2TY medium supplemented with 50 μg mL^{-1} ampicillin and 50 μg mL^{-1} chloramphenicol. Cells were grown until an OD_{600} of 0.6 – 0.7 was reached before cooling to 30 °C; expression was induced with isopropyl β-D-1-thiogalactopyranoside (0.5 mM final concentration). The cells were then incubated for a further four hours at 30 °C. Recombinant AmpC from P. aeruginosa[15], with an N-terminal His-tag, was produced in E. coli BL21(DE3) cells using auto-induction medium supplemented with 50 μg mL^{-1} ampicillin. Cells were grown for four hours at 37 °C before cooling to 18 °C and continuing growth overnight.

Cells were harvested by centrifugation (10 min, 10000 g), resuspended in 50 mL lysis buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 5 mM imidazole), supplemented with DNAse

I, then lysed by sonication. The supernatant was loaded onto a 5 mL HisTrap HP column followed by extensive washing with 50 mM HEPES, pH 7.5, 500 mM NaCl, 5 mM imidazole, before elution with a 20−500 mM imidazole gradient. Fractions containing purified enzyme were concentrated by centrifugal ultrafiltration (Amicon Ultra -15 mL, 10 kDa MWCO, Millipore). The resultant solution was injected onto a Superdex S200 column (300 mL) and eluted with 50 mM HEPES, pH 7.5, 200 mM NaCl. For AmpC and VIM-2, fractions containing pure His-tagged enzyme were incubated overnight at 4 °C with His-tagged 3C protease (1:100 w/w) to remove the N-terminal His-tag. The 3C protease together with any uncleaved protein the digestion mixture was removed by use of a second HisTrap HP column pre-equilibrated with 50 mM HEPES, pH 7.5, 500 mM NaCl, 20 mM imidazole. Chromatography employed using ÄKTAFPLC machine.

Purified enzyme containing fractions, as identified by SDS-PAGE, were pooled and concentrated by centrifugal ultrafiltration, then buffer exchanged into 25 mM HEPES, pH 7.5, 100 mM NaCl. The concentrations of the purified proteins were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, ε = 61310, or 31400 M^{-1} cm^{-1} for AmpC, or VIM-2, respectively).

Crystallisation Experiments, X-ray Data Collection and Processing

Crystallisation experiments were set up using a 18 mg mL^{-1} solution of AmpC in 50 mM HEPES, pH 7.5, 100 mM NaCl supplemented with 10 mM **1**. **1** and **2** were prepared as reported[16]. Crystallisation was performed at room temperature using the sitting drop vapour diffusion method. Crystals were obtained after approximately five months using 100 μL reservoir solution comprised of 200 mM $Zn(OAc)_2.2H_2O$, 100 mM imidazole, 20% PEG 3000, pH 8.0 and a 1:1 mixture (0.2 μL:0.2 μL) of protein to reservoir solution in the crystallisation drop. Crystals were cryo-protected using 25% glycerol in reservoir solution before harvesting with nylon loops and flash-cooling in liquid nitrogen. Diffraction data were collected at 100 K on beamline I04 of the Diamond Light Source, Didcot. Diffraction data were integrated and scaled using autoPROC. The structure was solved by isomorphous replacement using a published structure (PDB accession code: 4WYY) as a search model. The structure was then fit and refined iteratively using PHENIX[20] and Coot[21].

Surface Plasmon Resonance

A GE Healthcare Biacore T200 machine was used for all SPR experiments. The temperature was kept at 4 ^{o}C to increase stability of the protein on the chip. VIM-2 was ‘minimally’ biotinylated, i.e. protein was incubated with EZ link NHS-LCLC-Biotin (succinimidyl-6-

(biotinamido)-6-hexanamido hexanoate, ThermoScientific) at a 1:1 ratio for 2 hours at 4 °C; the excess of biotin was removed using a desalting column. The modified VIM-2 was attached to the streptavidin coated surface of the sensor chip in running buffer: 50 mM Hepes (pH 7.4), 150 mM NaCl, 0.05% (v/v) Tween 20, 3% (v/v) DMSO at ~ 3000 RU. The boronate inhibitor was screened at concentrations ranging from 45 nM – 3.7 uM, injecting from the lowest to highest concentrations. Kinetics were fitted using a 1:1 binding model with local Rmax for each concentration due to saturation of the surface.[ref] Data with the inhibitors were referenced to those for a blank surface and blank injections to normalize for non-specific binding and drift. A DMSO calibration was run to remove excluded volume effect of binding responses between reference and target surface.[ref] Binding was assayed at pH 6.5, 7.5 and 8.5 in 50 mM Hepes.

β-Lactamase Induction Experiments

Induction experiments were carried out as described[22]. In brief, cell cultures were grown overnight using nutrient broth and used to inoculate (1:100 dilution) 10 mL nutrient broth cultures. Cultures were incubated for 2 h with shaking at 37 °C before addition of potential inducers and growth for a further 2 h. Cells were pelleted by centrifugation (4000 g, 10 min) and were treated with 100 µL of BugBuster (Ambion). Cell debris was pelleted by centrifugation (13,000g, 5 min). Protein concentrations in the supernatant were determined using a BioRad protein assay reagent concentrate, according to the manufacturer's instructions. β-lactamase activity was determined using an Omega Fluostar (BMG Biotech) using meropenem as substrate.

Antimicrobial Susceptibility Testing

MICs were determined and interpreted for meropenem both alone, and in combination with **2** (10 µg/ml), by EUCAST/CLSI guidelines, in line with previously reported data[23].

Results

To investigate the structural basis of class C β-lactamase inhibition by bicyclic boronates we co-crystallised recombinant AmpC from P. aeruginosa in complex with bicyclic boronate **1**. Although crystals were only obtained after months, the overall fold of the AmpC-boronate **1** complex structure is very similar to that of an apo-AmpC structure (PDB accession code: 4GZB), with an RMSD of 0.325 Å over atoms in the peptide backbone and 0.251 Å for the residues interacting with the inhibitor at the active site. Analysis of non-protein electron density at the AmpC active site reveals that the bicyclic core of boronate **1** is intact and binds to AmpC via reaction of the nucleophilic serine (Ser90) with the boron of **1** to give a tetrahedral species. As observed with AmpC-Relebactam complex crystal structures, the amide nitrogen

and carbonyl group of the aminoacyl side chain are positioned to make hydrogen bonding interactions with active site residues (Gln146, Asn179 and Ser345),[24] while the saturated boracyclic ring is positioned beside tyrosine (Tyr249) and valine (Val239) apparently making hydrophobic interactions. The carboxylate group of **1** is directed towards the positively charged Lys342, while forming additional hydrogen bonding interactions with Thr343 and Asn373 (Figure 2), i.e. it binds in a similar manner to that predicted for the analogous carboxylates of AmpC/ class C β-lactamase substrates[25-26].

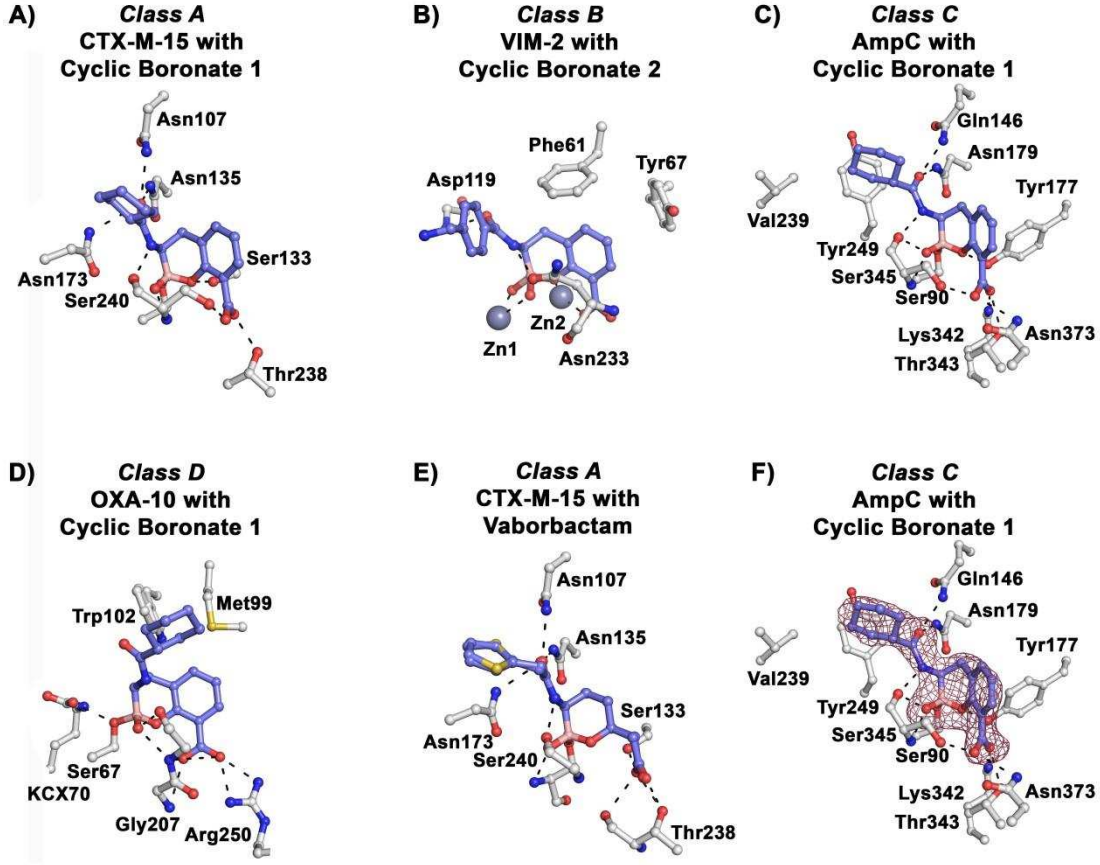

Figure 2: Structural basis of serine- and metallo-β-lactamase inhibition by cyclic boronates. The figure shows views from crystal structure of cyclic boronate 1/2 in complex with representatives from all four classes of β-lactamases (A to D). **A)** and **E)** Comparison of the binding modes of **1** (PDB ID: 5T66) and Vaborbactam (PDB ID: 4XUZ) observed with CTX-M-15. **B)** and **D)** View from a crystal structure of cyclic boronate **2** in complex with VIM-2 (PDB ID: 5FQC) and cyclic boronate **1** in complex with OXA-10 (PDB ID: 5FQ9). **C)** and **F)** View from a crystal structure of cyclic boronate **1** in complex with AmpC (PDB ID: 6I30). Representative electron density for **1** is shown (3.0 σ $_m$Fo-DF$_c$ OMIT, red mesh).

The bicyclic core of **1** binds to AmpC in a remarkably similar conformation / mode to that observed for the class A (CTX-M-15 and L2) and D (OXA-10) SBLs (Figure 2), i.e. via formation of a likely anionic tetrahedral species formed via reaction of the active site nucleophilic serine with the boron of **1** to produce a complex mimicking that proposed in β-

lactamase mediated hydrolysis of bicyclic β-lactams[15-16, 22]. The binding of the aryl carboxylate is similar in all cases, with differences reflecting the different residues involved in binding this substrate element in the different SBL types. A similar binding mode, including with respect to the observation of a tetrahedral boron has also been observed for **1**/**2** when complexed with PBP5 from Escherichia coli[16], though note that the tested bicyclic boronates tend to be much weaker PBP inhibitors (and antibiotics) than β-lactamase inhibitors[15-16] Thus, a conserved mode of binding is observed for bicyclic boronates with all three Ambler classes of serine-β-lactamase and, at least, one penicillin binding protein (PBP-5). Although, there are differences in the active site chemistry of the SBLs and MBLs, crystallographic analyses on the class B1 MBLs BcII and VIM-2[15-16] show that the conformation of the biyclic boronate **2** as observed in SBLs (and PBP5) is also maintained in binding to these clinically relevant MBLs[15-16] (Figure 2).

An overlay of our AmpC:**1** structure with a structure of AmpC from Enterobacter cloacae in complex with the 'monocyclic' boronate SBL inhibitor Vaborbactam (4XUX, Supplementary Figure S1)[27] indicates similar binding modes for the two compounds with their carboxylates groups being similarly positioned to interact with Lys342/335, Thr343/336 and Asn373/366, and the aminoacyl sidechain adopting similar conformations. The same relative positioning of the boron centre and a carboxylate moiety is seen in two structures of AmpC from P. aeruginosa in complex with 4,5-disubstituted oxaboroles (4WYY and 4WZ4)[28], although the aromatic carbocycle is oriented differently in these cases, likely due to the differently positioned carboxylate moiety in these molecules.

Reaction of an sp^2 boron, or substitution of an sp^3 B-OH, with the nucleophilic serine is necessary to form the crystallographically observed tetrahedral (sp^3) complex in the case of the SBLs/PBPs. However, for the MBLs either the sp^2 or sp^3 forms of the inhibitor could be envisaged to bind. To investigate binding of **2** to an MBL in solution, we used surface plasmon resonance (SPR) with the clinically relevant B1 MBL VIM-2. Data were collected at pH 6.5, 7.5, and 8.5 (Figure 3A and Supplementary Figure S1). The affinity of **2** for VIM-2, as measured by the K_D, varies by ~10 fold with pH, with the lowest K_D at pH 6.5 (4.97 nM) and a highest at pH 8.5 (55.3 nM), with the differences principally being due to changes in the association rate (k_{on}). At least in part this may reflect a bias to the sp^2 rather than the sp^3 hybridisation states of the boron of the inhibitor at lower pH[29], though other factors including potential ring opening/closing of the inhibitor (Figure 1B) and the β-lactamase protonation state may be relevant, these observations are consistent with the sp^2 form of the inhibitor reacting with the zinc ion activated hydrolytic water / hydroxide at the VIM-2 active site.

Treatment of bacteria with β-lactam antibiotics can induce expression of chromosomal genes for β-lactamases, for example AmpC enzymes[30]. This upregulation can arise as a result of signalling due to inhibition of cell wall biosynthesis by β-lactam antibiotics[30]. Administration of all clinically used β-lactamase inhibitors results in upregulated β-lactamase production[2, 31], hence β-lactamase inhibitors that do not induce such an effect may useful in a clinical setting. We have reported that both **2** and avibactam do not manifest detectable β-lactamase induction in S. maltophilia[22]. Recent reports have demonstrated that the non β-lactam, but serine modifying inhibitor, avibactam can induce the AmpC β-lactamase in Enterobacteriaceae, including E. cloacae and some P. aeruginosa[31]. Thus, we investigated the effects of **2,** which has a different mode of action, on β-lactamase induction in E. cloacae and P. aeruginosa. As anticipated[22, 31], with both E. cloacae and P. aeruginosa treatment with a cephalosporin, carbapenem, or clavulanic acid results in an increased production of β-lactamases even at 10 μg mL^{-1} (Figure 3B). By contrast, avibactam or **2** does not induce β-lactamase production within limits of detection with either P. aeruginosa or E. cloacae even at a 50 μg mL^{-1} (Figure 3).

A)

pH	k_{on} ($m^{-1}Ms^{-1}$)	k_{off} (s^{-1} x 10^{-4})	K_D(nM)
6.5	175.1	8.71	4.97
7.5	58.77	6.13	10.43
8.5	8	4.44	55.3

C)

Species	MIC_{50}(μg/ml)		MIC_{90}(μg/ml)	
	MEM	MEM+2	MEM	MEM+2
Total Enterobacteriaceae (n=134)	32	2	>64	>64
Enterobacter spp. (n=21)	8	2	64	8
E. Coli (n=25)	16	2	64	64
K. Pneumoniae (n=78)	32	2	>64	64

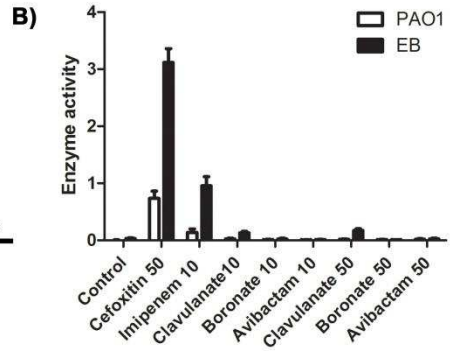

Figure 3: Binding and microbiological studies with 2. A) Fitted constants for the binding of **2** to VIM-2 as determined by SPR.; **B)** Induction of β-lactamase production by cephalosporins, carbapenems and representative β-lactamase inhibitors as measured by relative activity. (PAO – Pseudomonas aeruginosa and EB – Enterobacter cloacae); C) $MIC_{50/90}$s of Antimicrobial Test Panel vs Ambler Class B (Metallo) β-Lactamases-producing Enterobacteriaceae by Species (MEM – meropenem).

We then investigated the activity of **2** in combination with meropenem against contemporary, clinical NDM (n=104) and VIM-positive (n=28) Enterobacteriaceae, because these B1 MBLs catalyse the hydrolysis of a broad range of β-lactams, including carbapenems (not monobactams). **2** significantly improved the rates of meropenem susceptibility (MIC ≤ 2 μg mL^{-1}) against MBL-positive Enterobacteriaceae. Meropenem susceptibility rates of MBL-positive E. coli with and without **2** were 8% and 64% respectively (Figure 3C and Figures S3-5). The corresponding susceptibility rates for MBL-positive Klebsiella pneumoniae were 8%

and 55% respectively. NDM-1-positive A. baumannii (n=3) and VIM-positive P. aeruginosa (n=2) were also tested; **2**, however, did not induce an MIC shift for meropenem in this case.

Conclusions

Boronic acids have been known to inhibit SBLs since the 1970s[32] and have a much longer history as antibaterials (since 1880s)[33]. Vaborbactam, an inhibitor of class A, C, and some class SBLs, has been introduced for clinical use; however, there is, at present no clinically available MBL inhibitors based on the boronic acid chemotype. Bicyclic boronates of the type characterised here and in our previous work[15-16, 22] hold potential as broad spectrum SBL/MBL inhibitors. Together, with work on CTX-M-15, L2, BcII, VIM-2 and OXA-10 β-lactamases[15-16, 22] the crystallographic work presented here on AmpC, a commonly expressed class C β-lactamase in resistant P. aeruginosa-based infections, reveals that it is possible to inhibit (representative) β-lactamases from all four Ambler classes by a single compound type operating via a common mechanism, i.e. mimicking the tetrahedral intermediate common to SBLs and MBLs. Moreover, comparison of our AmpC:**1** structure with that of Vaborbactam with AmpC[27], reveals highly similar binding modes for the boron containing ring, aryl-carboxylate, and N-acetamido acid chain.

Given the clear differences in spectrum of activities for Vaborbactam and biyclic boronates, with the latter in general appearing better in vitro, there appear to be scope for improving the activity of boronates by extending the rather limited SAR reported to date (at least compared to the enormous studies on β-lactam antibiotics / β-lactamase inhibitors). The bicyclic boronates studied here and in our prior work closely resemble VNRX-5133[34] (Figure 1C), which has not been profiled for MBL coverage. Further studies on the precise binding modes of the boronates to β-lactamases and PBPs are of interest, including with respect to increasing their potency versus PBPs and broadening the scope of MBL inhibition (our compounds show only limited inhibitory activity against certain MBLs, including IMP-1, SPM-1, CphA, and L1[1622]. Our preliminary studies suggest that binding to both SBLs and MBLs may involve the sp^2 inhibitor form (Figure 1B), again reinforcing the proposed similarity (20,21) in binding mode of the bicyclic boronates for SBLs and MBLs.

SBL inhibition by clavulanic acid and related β-lactams (tazobactam, sulbactam) is proposed to occurs via acy-enzyme fragmentation resulting in inactivation[35]. By contrast, avibactam, inhibits SBLs via reversible covalent binding[35]. As revealed by crystallography, the binding modes of bicyclic boronates, resemble those of intermediates an route to the acyl-enzyme

complexes formed with β-lactam antibiotics. The boronate inhibitors also exist as an equilibrium between sp^2/sp^3 states. Thus, it is unclear to what extent borontes will mirror β-lactams in terms of their β-lactamase inducing capacity. Our results show that **2** does not induce β-lactamase production within detection limits in the tested organisms at up to 50 μg mL^{-1}. By contrast, avibactam induces β-lactamase production in some P. aeruginosa but not in E. cloacae[31]. The lack of induced β-lactamase production by **2**, and potentially other boronates, may in part reflect the good clinical coverage observed with co-dosing of **2** with meropenem, which clearly increases the meropenem susceptibility against MBL-positive Enterobacteriaceae. Further structure activity relationship work on the ability of β-lactamases inhibitor templates to induce b-lactamases are thus of interest.

Acknowledgements

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