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

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Abstract

Background

The β-lactam antibiotics represent the most successful drug 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- (SBL) and metallo- (MBL) β-lactamase families.

Methods

Using biophysical methods, including crystallographic analysis, we have investigated the binding mode of bicyclic boronates to clinically important β-lactamases. Induction experiments and agar-based MIC screening against MDR-*Enterobacteriaceae* (n=132) were used to evaluate induction properties and the *in vitro* efficacy of a bicyclic boronate in combination with meropenem.

Results

Crystallographic analysis of a bicyclic boronate in complex with AmpC from *Pseudomonas aeruginosa* reveals it binds to form a tetrahedral boronate species. 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.

Conclusions

Together with reported studies on the structural basis of their inhibition of class A, B and D β-lactamases, biophysical studies, including crystallographic analysis, support the proposal that bicyclic boronates mimic tetrahedral intermediates common to SBL and MBL catalysis.

General significance

Bicyclic boronates are a new generation of broad spectrum inhibitors of both SBLs and MBLs.

Keywords: β-lactam antibiotic resistance, cyclic boronate inhibitors, metallo and serine β-lactamase inhibition, transition state analogue, β-lactamase induction, antimicrobial clinical coverage.

Abbreviations:

SBLs – serine-β-lactamases

MBLs – metallo-β-lactamases

cUTI – complicated urinary tract infections

PAO – *Pseudomonas aeruginosa*

EB – *Enterobacter cloacae*

MEM – meropenem.

1. Introduction

The β-lactam antibiotics represent the most successful drug class for treatment of bacterial infections[1]. Resistance mechanisms, particularly involving 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 achieved in the treatment of bacterial infections exhibiting resistance by some serine-β-lactamases (SBLs), particularly Ambler class A enzymes, *via* co-administration 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 may be 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 1B), 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 some clinically relevant SBLs[10].

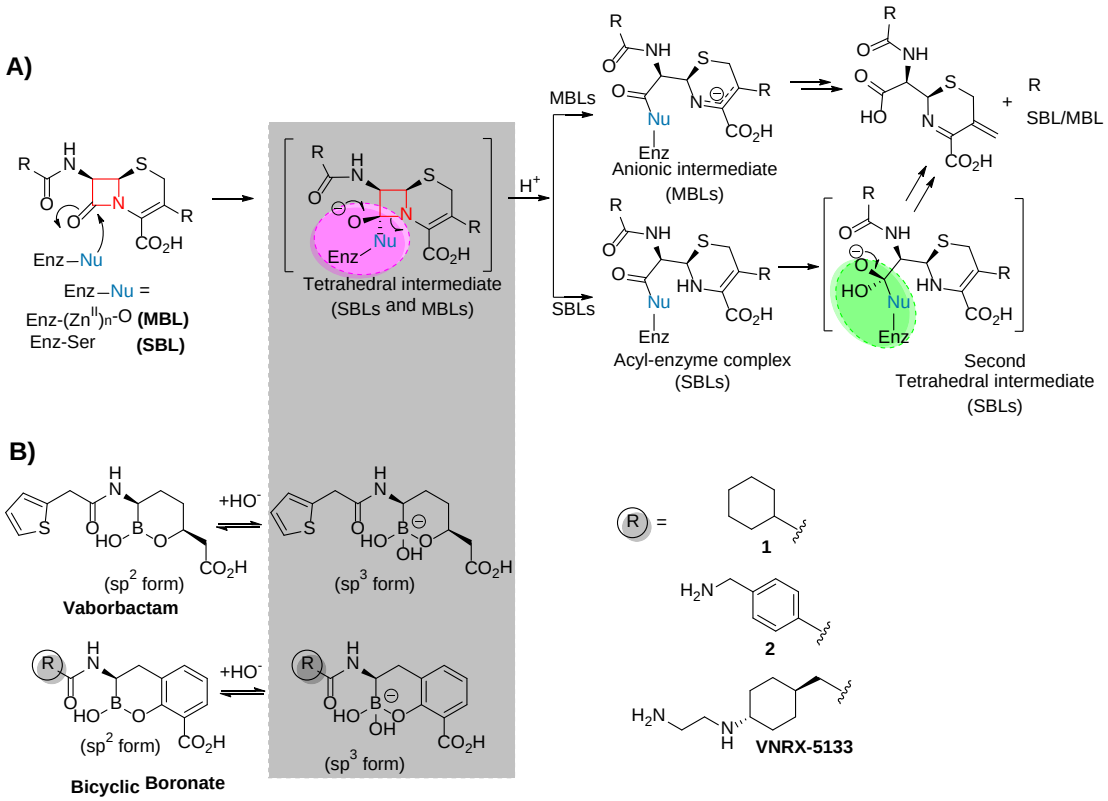

Figure 1. A) Outline mechanisms of serine- and metallo-β-lactamase (SBL and MBL) catalysis, exemplified by hydrolysis of a cephalosporin. Note that products can be produced in different tautomeric forms. The tetrahedral intermediate, common to both SBLs and MBLs,

may be mimicked by the sp^3 form of cyclic boronates. **B)** Structures of Vaborbactam and the bicyclic boronate β-lactamase inhibitors VNRX-5133, **1**, and **2**.

In contrast to 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 structurally heterogeneous within their own class (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], in addition to SBL-mediated resistance to avibactam, suggests that future use of avibactam may be compromised by β-lactamases[13, 14]. Thus, the development of dual-action SBL and MBL β-lactamase inhibitors is of interest.

We have reported that boronates with a bicyclic scaffold, at least predominantly in solution, 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[15, 16] and other biophysical analyses our results support the proposal that bicyclic boronates mimic the tetrahedral intermediates common to both SBL and MBL 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.

2. Materials and Methods

2.1. Enzyme production

Recombinant VIM-2, with an *N*-terminal His-tag, was produced using the reported pOPINF vector[18] in *Escherichia 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, then 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 in 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 used an ÄKTA FPLC 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. 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).

2.2. Crystallisation Experiments, X-ray Data Collection and Processing

Crystallisation experiments were set up using an 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% (*v*/*v*) final glycerol in the reservoir solution before harvesting with nylon loops and flash-cooling in liquid nitrogen. Diffraction data were collected at 100 K at 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 reported AmpC structure (PDB accession code: 4WYY) as a search model. The structure was then fit and refined iteratively using PHENIX[19] and Coot[20].

2.3. Surface Plasmon Resonance

A GE Healthcare Biacore T200 machine was used for all SPR experiments. The temperature was kept at 4 °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 13 nM – 10 µM, injecting from the lowest to highest concentrations. Kinetics were fitted using a 1:1 binding model with local R_{max} for each concentration due to saturation of the surface. Data with the inhibitors were referenced to those for a blank surface and blank injections to normalize for non-specific binding and drift. Single concentration series was run for each dataset. A DMSO calibration was run to remove excluded volume effect of binding responses between reference and target surface. Binding was assayed at pH 6.5, 7.5 and 8.5 in 50 mM HEPES.

2.4. ^{11}B-NMR Spectroscopy

^{11}B-NMR spectra were acquired using a Bruker AVIIIHD 600 MHz spectrometer equipped with a Prodigy N2 broadband cryoprobe. Experiments were performed at 298 K, with an acquisition time of 0.85 s and a relaxation delay of 0.5 s. Spectra consisted of 4096 scans and were processed with a line broadening of 5.0 Hz and manual multipoint baseline correction. The impact of pH was shown using 200 µM **2** in 50 mM sodium phosphate (pH 7.5 or pH 12.0) or 50 mM sodium acetate (pH 4.5), supplemented with 10 % D_2O. Samples were measured in thin-walled quartz NMR tubes (5 mm O.D.; VWR).

2.5. β-Lactamase Induction Experiments

Induction experiments were carried out as described[21]. 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 hours with shaking at 37 °C before addition of potential inducers and growth for a further 2 hours. Cells were pelleted by centrifugation (4000 g, 10 min) and were treated with 100 µL of BugBuster (Ambion). Cell debris was pelleted by centrifugation (13,000*g*, 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) machine with meropenem (Glentham Life Sciences) as a substrate.

2.6. Antimicrobial Susceptibility Testing

Meropenem (MEM) was tested alone (0.06-64 µg mL^{-1}) and in combination with boronate **2** (10 µg mL^{-1}) against a collection of clinical, carbapenemase-producing *Enterobacteriaceae* (Supplementary Table S4). These strains represent a global collection originating from studies in India, Pakistan, Egypt and Spain. MICs were determined by agar dilution, and interpreted using published guidelines described by EUCAST/CLSI[22].

2.7. Binding mode of VNRX-5133

To investigate the structural basis of VNRX-5133 interaction with the MBLs, a model of VNRX-5133 with the B1 MBL VIM-2, based upon the binding mode of a bicyclic boronate (PDB ID: 5FQC) was constructed. Studies were conducted using AutoDock 4.21 (http://autodock.scripps.edu) and the active site was defined using Maestro (https://www.schrodinger.com/maestro).

The *in silico* docking studies conducted using AutoDock 4.21 were performed as described in the steps below:

1) Coordinates for a crystal structure of VIM-2 were obtained from the Protein Databank (PDB ID: 5FQC)[16].
2) The active site was defined using Maestro.3 with 20 Å region of the protein surrounding the active site was designated as the protein receptor region.
3) VNRX-5133 was constructed in Maestro.3 with coordinates to both zinc ions. (The boron atom in VNRX-5133 was substituted for a carbon atom due to the limited parameters available for boron within AutoDock 4.2; the introduced carbon manifests a similar tetrahedral geometry compared to the bound boron). Next, the ligand was then fully energy minimised using the multiple minimisation tool (MM in AutoDock 4.2).

4) VRNX-5133 was assigned as a flexible residue.
5) Water was docked into the active site of VIM-2 using AutoDock 4.2 (a box of 50 x 50 x 50 Å was used) which utilises a Lamarckian genetic algorithm. Fifty dockings were conducted for each compound. The use of the flexible residue setting allows VRNX-5133 to move within the active site with the constraint of ligation to the zinc ions.
6) The resulting docking poses were visually inspected using AutoDockTools.

3. Results

3.1. AmpC Crystal Structure with 1 Informs on the Structural Basis of Pan-β-Lactamase Inhibition

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** (Figure 2.C and E). Although crystals were only obtained after months, the overall fold of the AmpC-boronate **1** complex structure is very similar to that of a reported apo-AmpC structure (PDB accession code: 4GZB)[23], with an RMSD of 0.325 Å for peptide backbone atoms 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 indicates 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 (sp^3 hybridised) species. As observed with AmpC-Relebactam complex crystal structures[24], the amide nitrogen and carbonyl group of the inhibitor aminoacyl side chain are positioned to make hydrogen bonding interactions with active site residues (Gln146, Asn179 and Ser345)[25], 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, and is positioned to form 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[24, 26].

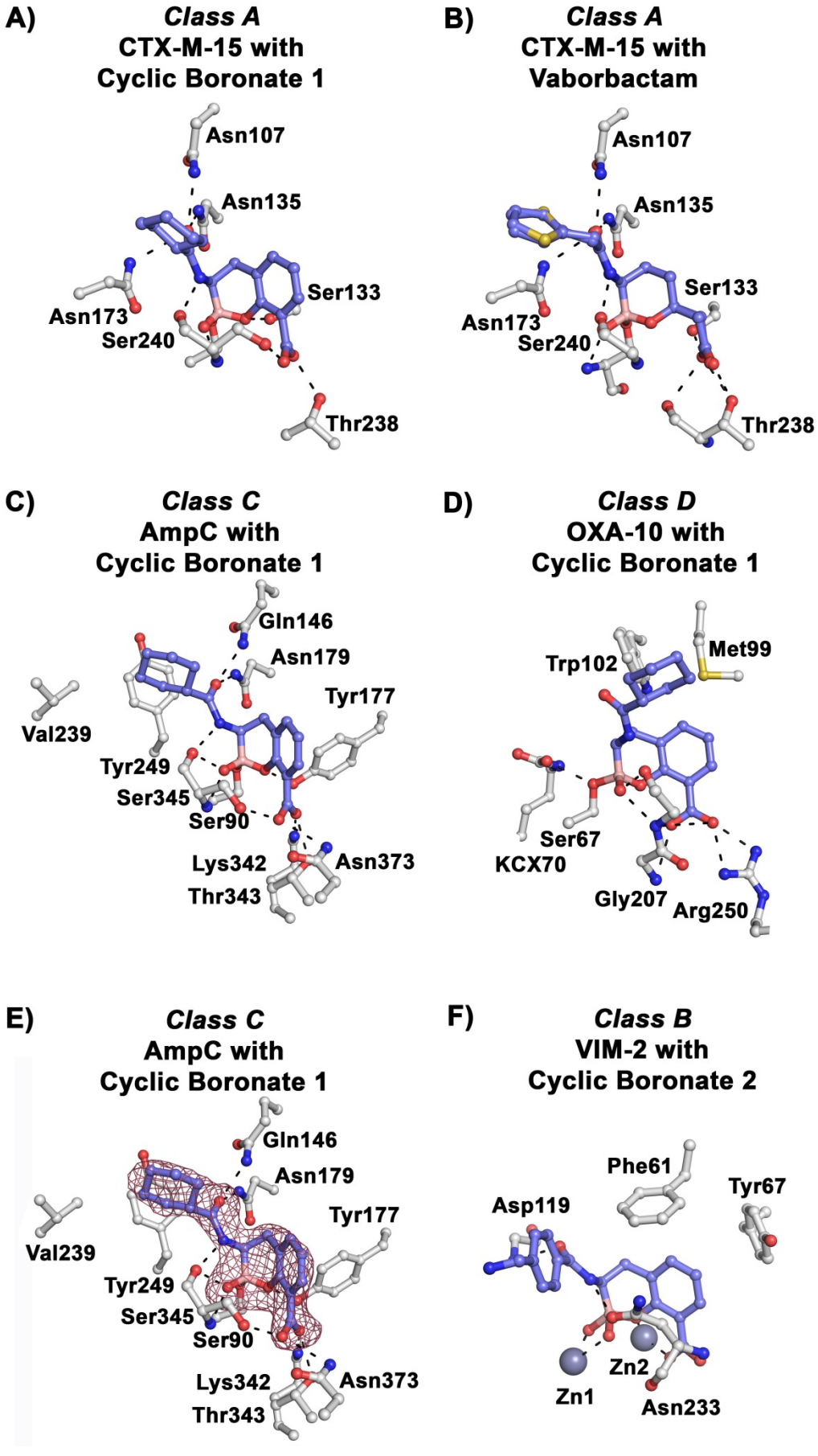
A)
Class A
CTX-M-15 with
Cyclic Boronate 1
Asn107
Asn135
Ser133
Asn173
Ser240
Thr238
B)
Class A
CTX-M-15 with
Vaborbactam
Asn107
Asn135
Ser133
Asn173
Ser240
Thr238
C)
Class C
AmpC with
Cyclic Boronate 1
Gln146
Asn179
Tyr177
Val239
Tyr249
Ser345
Ser90
Lys342
Asn373
Thr343
D)
Class D
OXA-10 with
Cyclic Boronate 1
Met99
Trp102
Ser67
KCX70
Gly207
Arg250
E)
Class C
AmpC with
Cyclic Boronate 1
Gln146
Asn179
Tyr177
Val239
Tyr249
Ser345
Ser90
Lys342
Asn373
Thr343
F)
Class B
VIM-2 with
Cyclic Boronate 2
Phe61
Tyr67
Asp119
Zn2
Zn1
Asn233

Figure 2: Structural basis of serine- and metallo-β-lactamase inhibition by cyclic boronates. Views from crystal structures of cyclic boronates **1**/**2** in complex with representatives from all four classes of β-lactamases (A to D)[15, 16]. **A)** and **B)** Comparison of the binding modes of **1** (PDB ID: 5T66) and Vaborbactam (PDB ID: 4XUZ) observed with CTX-M-15. **C)** and **D)** Comparison of the binding modes of **1** in complex with AmpC (PDB ID: 6I30) and with OXA-10 (PDB ID: 5FQ9); **E)** 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 $_{m}F$o-DF_{c} OMIT, red mesh); and **F)** View from a crystal structures of cyclic boronate **2** in complex with VIM-2 (PDB ID: 5FQC)[16].

The bicyclic core of **1** binds to AmpC in a remarkably similar conformation to those observed for the class A (CTX-M-15[15] and L2[21]) and D (OXA-10[16]) 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, 21]. 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 various 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 penicillin binding protein-5 (PBP-5) from *Escherichia coli* [16], though note that, to date, tested bicyclic boronates tend to be weaker PBP inhibitors (and hence antibiotics) than β-lactamase inhibitors[15, 16]. Thus, a conserved mode of binding is observed for bicyclic boronates with all three Ambler classes of SBL 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 bicyclic 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 carboxylate 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 atom and the carboxylate group is seen in two structures of AmpC from *P. aeruginosa* in complex with 4,5-disubstituted oxaboroles (4WYY and 4WZ4)[28]; however, the aromatic ring is oriented differently in these cases, likely due to the differently positioned carboxylate moiety in these molecules.

3.2. NMR and Surface Plasmon Resonance Studies

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) complexes in the case of the SBLs/PBPs[15, 16, 29]. In solution **2** or other boronates exist preferentially in the sp^2 hybridisation state at pH 4.5 and 7.4 and in the sp^3 hybridisation at pH 10 as shown by ^{11}B NMR (Supplementary Figure S3)[30, 31]. These observations support initial binding *via* the sp^2 hybridisation state. However, for the MBLs, either the sp^2 or sp^3 forms of the inhibitor could be envisaged to bind.

Table 1: Fitted constants for the binding of 2 to VIM-2 as determined by SPR

pH	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (nM)	Concentration series
6.5	1.62 (±0.03)x10^5	2.35 (±0.02)x10^{-3}	14.5 (±0.2) nM	13.7-370 nM
7.5	2.70 (±0.02)x10^4	6.50 (±0.03)x10^{-4}	24.0 (±0.2) nM	13.7nM – 3.3uM
8.5	8.00 (±0.10)x10^3	4.44 (±0.04)x10^{-4}	55 (±0.1) nM	41nM – 10 uM

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 K_D, varies with pH, with the lowest K_D at pH 6.5 (14.5 nM) and a highest at pH 8.5 (55 nM). Differences principally occur as a result of changes in the association rate ($k_{a,}$, Table 1). At least in par,t 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 values (Supplementary Figures S3)[32], 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 binding reacting with a zinc ion activated hydrolytic water/hydroxide at the VIM-2 active site.

3.3. Induction Experiments Reveal no Detectable β-Lactamase Production with Bicyclic Boronate 2

Treatment of bacteria with β-lactam antibiotics can induce expression of chromosomal β-lactamase such as AmpC[33]; this upregulation can arise as a result of signalling due to inhibition of cell wall biosynthesis induced by β-lactam antibiotics[33]. Administration of all clinically used β-lactamase inhibitors results in upregulated β-lactamase production [2, 34]; hence β-lactamase inhibitors that do not, or manifest reduced apparent induction of β-lactamases are of interest.

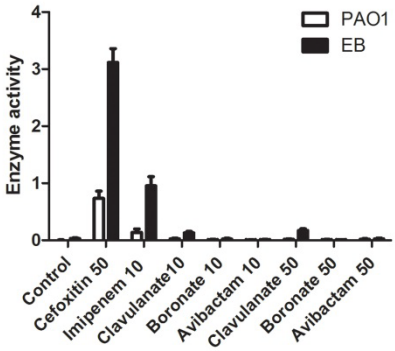

Figure 3: Induction of β-lactamase production. Induction of β-lactamase production by cephalosporin, carbapenem, and representative β-lactamase inhibitors as measured by relative activity (PAO – *P. aeruginosa* and EB – *E. cloacae*);

We have reported that both **2** and avibactam (a non β-lactam inhibitor) did not manifest detectable β-lactamase induction (as measured by activity assays) in *S. maltophilia*[21]. Recent reports have demonstrated that avibactam induces the AmpC β-lactamase in Enterobacteriaceae, including *E. cloacae* and some *P. aeruginosa* strains[34]. Thus, we investigated the effects of **2**, which has a different mode of action, on β-lactamase induction/activity in *E. cloacae* and *P. aeruginosa*. As anticipated[21, 34], with both *E. cloacae* and *P. aeruginosa* treatment with a cephalosporin, carbapenem, or clavulanic acid results in observation of increased β-lactamase activity even at 10 μg mL^{-1} (Figure 3B). By contrast, neither avibactam nor **2** induced apparent β-lactamase production, as measured by nitrocefin hydrolysis, within our limits of detection with either *P. aeruginosa* or *E. cloacae*, even at a 50 μg mL^{-1} (Figure 3). Note, we cannot rule out that residual **2**/avibactam may be (partially) inhibiting induced β-lactamase activity.

3.4. Microbiology Experiments Confirm the Good Clinical Coverage of 2 in Combination with Meropenem

We then investigated the activity of **2** (a superior MBL inhibitor compared to **1**)[16] in combination with meropenem against contemporary, clinical NDM (n=104) and VIM-positive (n=28) *Enterobacteriaceae* strains, because these B1 MBLs catalyse the hydrolysis of a broad range of β-lactams, including carbapenems (not monobactams).

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

Table 2: Microbiology analyses with boronate 2. $MIC_{50/90}$s of Antimicrobial Test Panel vs Ambler Class B (metallo) β-Lactamases-producing Enterobacteriaceae(MEM – meropenem)

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 (Table 2 and Supplementary Tables S5 and S6, and Figure S7). 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 observed MIC shift for meropenem in these cases.

4. Conclusions

Boronic acids have been known to inhibit SBLs since the 1970s[35] and have a much longer history as antibacterials (since the 1880s)[36]. Vaborbactam, an inhibitor of class A and C SBLs, has been introduced for clinical use; however, there are, at present, no clinically available MBL inhibitors based on the boronic acid chemotype. Together, with work on the CTX-M-15, L2, BcII, VIM-2, and OXA-10 β-lactamases[15, 16, 21], 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 the spectrum of activities for Vaborbactam and bicyclic boronates, with the latter in general appearing to act as better β-lactamase inhibitors *in vitro*, there seems 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[15, 16] closely resemble VNRX-5133[37] (Figure 1B and Supplementary Figure S6), for which MBL coverage has not yet been reported. 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)[16, 21]. Our preliminary studies suggest that initial binding to both SBLs and MBLs may involve the sp^2 hybridised inhibitor form, which better mimics the non-reacted β-lactam than the sp^3 hybridised form, as observed in the final enzyme-inhibitor complexes. The overall results thus reinforce the proposed similarity in binding mode of the bicyclic boronates for SBLs and MBLs.

SBL inhibition by clavulanic acid and, potentially, related β-lactamase inhibitors (tazobactam, sulbactam) is proposed to occur *via* acyl-enzyme fragmentation resulting in inactivation[38]. By contrast, avibactam, inhibits SBLs *via* reversible covalent binding[38]. As revealed by crystallography, the binding modes of bicyclic boronates, resemble those of the proposed tetrahedral intermediates *en route* to the acyl-enzyme complexes formed with β-lactam antibiotics.

The boronate inhibitors including **2** can exist in sp^2 or sp^3 hybridisation states in solution with the relative amounts depending on the pH (Supplementary Figure S3). Thus, it is unclear to what extent boronates 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 for 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*[34]. The lack of induced β-lactamase production by **2**, and potentially other boronates, may in part be reflected in the good clinical coverage observed with co-dosing of **2** with meropenem, which clearly increases meropenem susceptibility against MBL-positive *Enterobacteriaceae*. Further structure activity relationship work on the ability of non-β-lactam β-lactamase inhibitor templates to induce β-lactamases is thus of interest.

PBP ID

Coordinates and structure factors have been deposited in the Protein Data Bank. PDB ID is 6I30 for the crystal structure of cyclic boronate **1** in complex with AmpC from *Pseudomonas aeruginosa*.

Competing interest

The authors declare that there are no competing interests.

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Appendix A. Supplementary data.

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