Resistance to aztreonam, in combination with a bicyclic boronate β-lactamase inhibitor in *Escherichia coli* identified following mixed culture selection.

Ching Hei Phoebe Cheung[1], Jacqueline Findlay[1], Kate J. Heesom[2] & Matthew B. Avison[1]

[1]School of Cellular & Molecular Medicine, University of Bristol. UK

[2]Bristol University Proteomics Facility, University of Bristol. UK.

Abstract

Background

Bicyclic boronates are a new and potentially important class of β-lactamase inhibitor, with the ability to inhibit β-lactamases from all molecular classes, including mobile metallo-β-lactamases.

Objective

Our objective was to identify mutants resistant to the actions of the bicyclic boronate inhibitor **2**, when being used in combination with aztreonam.

Methods

Overnight cultures were plated on to agar containing increasing concentrations of aztreonam with a fixed 10 mg/L concentration of the inhibitor. Resistant derivatives and parent strains were analysed by whole genome sequencing and LC-MS/MS proteomics to identify mechanism of resistance.

Results

When using a mixed overnight culture containing one *Escherichia coli* (TEM-1, CTX-M-15, CMY-4 producer) and one *Klebsiella pneumoniae* (SHV-12, CTX-M-15, NDM-1 producer) mobilisation of an IncX3 plasmid carrying bla_{SHV-12} from the *K. pneumoniae* into the *E. coli* generated an aztreonam/boronate resistant derivative.

Conclusions

High-level production of three bicyclic boronate susceptible enzymes (CMY-4, CTX-M-15, SHV-12) capable of hydrolysing aztreonam plus TEM-1, which binds the inhibitor, overcomes the fixed inhibitor dose used. This was only identified when

using a mixed culture for selection. It would seem prudent that to allow for coalescence of the myriad β-lactamase genes commonly found in bacterial populations colonising humans, this mixed culture approach should be the norm when testing the potential for generating β-lactamase inhibitor resistance in pre-clinical analysis.

Introduction

β-Lactamase enzymes, which are divided into molecular classes A, B, C and D, catalyse hydrolysis of the four-membered ring in β-lactam antimicrobials. This renders the antimicrobial inactive, because the opened ring can no longer act as an inhibitor of peptidoglycan transpeptidases.[1] As β-lactams are the most widely used class of antimicrobial world-wide,[2] the dissemination of β-lactamases is threatening the efficacy of these drugs and is causing great concern. Indeed, β-lactamase activity is the most common β-lactam resistance mechanism in Gram-negative bacteria.[1] β-Lactamase inhibitors are therefore being used to overcome their effects.[3] The β-lactam-based class A β-lactamase inhibitors clavulanic acid and tazobactam are widely administered with penicillin derivatives and have had decades of clinical success. However, there are many β-lactamases that are not affected by these inhibitors, and there is particularly a lack of effective inhibitors for class B, zinc-dependent metallo-β-lactamases, which cause resistance to a wide range of β-lactams including penicillins, cephalosporins and carbapenems.[3] Non-β-lactam based β-lactamase inhibitors recently introduced into clinical practice are avibactam and vaborbactam, and these have a wider spectrum of activity than clavulanic acid or tazobactam, but they do not inhibit class B enzymes.[3] There have been several studies investigating the use of non-β-lactam boronic acid compounds as inhibitors, as they can form analogues of the transient oxyanionic intermediate species of β-lactamase hydrolysis. Vaborbactam is one of these,[3] but Brem et al. have shown that bicyclic boronates are potent inhibitors of both serine-β-lactamases (classes A, C and D) and the most common subclass of metallo-β-lactamases, subclass B1.[4] Another bicyclic boronate cross-class β-lactamase inhibitor, VNRX-5133 is shortly to be entering phase 3 clinical trials.[5]

We have been attempting to identify mutants resistant to the actions of the bicyclic boronate β-lactamase inhibitor **2** with very limited success. We report here one successful "mutant" selection which occurred following the accidental mixing of two isolates prior to selection. This experience has helped us to (a) understand how bicyclic boronate resistance may occur, and (b) realise that selecting β-lactam/β-lactamase inhibitor resistant derivatives from mixed cultures should be a routine approach in the future.

Material and Methods

Bacterial isolates and materials

Both parent isolates were from bloodstream infections in humans and were gifts from Prof Tim Walsh, Cardiff University. Growth media were from Oxoid, chemicals were from Sigma unless otherwise stated. The bicyclic boronate inhibitor **2** was synthesized according to the literature protocol [4] and kindly provided by Prof. C. Schofield, University of Oxford.

Fluorescent Hoescht (H) 33342 dye accumulation assay

Envelope permeability in living bacteria was tested using a standard dye accumulation assay protocol [6] where the dye only fluoresces if it crosses the entire envelope and interacts with DNA. Overnight cultures in LB Broth (LB) at 37°C were used to prepare Mueller-Hinton Broth (MHB) subcultures, which were incubated at 37°C until an optical density at 600 nm (OD_{600}) reached 0.6. Cells were pelleted by centrifugation (4000 rpm, 10 min) (ALC, PK121R) and resuspended in 500 µL of PBS. The optical densities of all suspensions were adjusted to 0.1 OD_{600}. Aliquots of 180 µL of cell suspension were transferred to a black flat-bottomed 96-well plate (Greiner Bio-one, Stonehouse, UK). Eight technical replicates, for each strain tested,

were in each column of the plate. The plate was transferred to a POLARstar spectrophotometer (BMG Labtech) and incubated at 37°C. Hoescht dye (H33342, 250 µM in water) was added to bacterial suspension of the plate using the plate-reader's auto-injector to give a final concentration of 25 µM per well. Excitation and emission filters were set at 355 nm and 460 nm respectively. Readings were taken in intervals (cycles) separated by 150 s. 31 cycles were run in total. A gain multiplier of 1460 was used. Results were expressed as absolute values of fluorescence versus time.

Proteomics

500 µL of an overnight LB culture were transferred to 50 mL MHB and cells were grown at 37˚C to 0.6 OD_{600}. Cells were pelleted by centrifugation (10 min, 4,000×g, 4°C) and resuspended in 30 mL of 30 mM Tris-HCl, pH 8 and broken by sonication using a cycle of 1 s on, 1 s off for 3 min at amplitude of 63% using a Sonics Vibracell VC-505TM (Sonics and Materials Inc., Newton, Connecticut, USA). The sonicated samples were centrifuged at 8,000 rpm (Sorval RC5B PLUS using an SS-34 rotor) for 15 min at 4°C to pellet intact cells and large cell debris. Protein in the supernatant was concentrated using an Amikon Ultra-15 centrifugal filter with an ultracel-3 membrane (Mercl) by centrifugation at 4000 rpm until the supernatant volume reduced to approximately 1 mL. Protein concentrations in all samples were quantified using Biorad Protein Assay Dye Reagent Concentrate according to the manufacturer's instructions. Proteins (1 µg/lane) were separated by SDS-PAGE using 11% acrylamide, 0.5% bis-acrylamide (Biorad) gels and a Biorad Min-Protein Tetracell chamber model 3000X1. Gels were resolved at 200 V until the dye front had moved approximately 1 cm into the separating gel. Proteins in all gels were stained with Instant Blue (Expedeon) for 20 min and de-stained in water. LC-MS/MS

data was collected as previously described.[7] The raw data files were processed and quantified using Proteome Discoverer software v1.4 (Thermo Scientific) and searched against bacterial genome and horizontally acquired resistance genes as described previously.[8]

Whole genome sequencing and data analysis

Genomes were sequenced by MicrobesNG (https://microbesng.uk/) on a HiSeq 2500 instrument (Illumina, San Diego, CA, USA) using 2x250 bp paired end reads. Reads were trimmed using Trimmomatic[9], assembled into contigs using SPAdes 3.13.0 (http://cab.spbu.ru/software/spades/) [10] and contigs were annotated using Prokka.[11] Plasmid replicon types, resistance genes and sequence types were determined using the PlasmidFinder,[12] ResFinder,[13] and MLST 2.0 [14] using the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/) platform.

Results and Discussion

A mixture of two human clinical isolates – one *E. coli* ST101 and one *K. pneumoniae* ST265 - was mistakenly inoculated into the same bottle containing Muller Hinton Broth and grown overnight without any antibiotic selection. The two isolates had previously been characterised separately using whole genome sequencing. **Table 1** lists the resistance gene and plasmid replicon carriage status of the isolates. One hundred microlitres of the mixed overnight culture was plated onto Muller Hinton agar containing aztreonam at increasing concentrations plus the bicyclic boronate **2** at a fixed concentration of 10 mg/L, as used previously.[4] Unexpectedly, profuse growth was seen on all plates up to 16 mg/L aztreonam, which is defined as resistant by CLSI. Multiple colonies were picked onto Tryptone Bile Glucuronic Agar and all

were confirmed to be *E. coli*. One aztreonam/inhibitor resistant derivative was selected as representative.

Envelope permeability assays showed that the parent *K. pneumoniae* used to make the mixed culture was less permeable than the parent *E. coli* but the resistant *E. coli* derivative behaved similarly to the parent *E. coli* (**Figure 1**). This was expected because proteomic analysis of key porin and efflux pump protein abundance showed the parent and resistant *E. coli* derivative were not significantly different (**Table 1**). Whole genome sequencing revealed that the complement of β-lactamases and plasmid replicon types in the resistant *E. coli* derivative had increased compared with the parent; an SHV-12 encoding IncX3 plasmid had clearly moved from the *K. pneumoniae* parent isolate into the *E. coli* parent isolate during co-culture. This plasmid does not carry any other resistance genes not already present in the *E. coli* parent isolate (**Table 1**). Proteomics of the *E. coli* derivative confirmed that this SHV-12 was expressed at high levels, and that the abundance of the other β-lactamases carried by the *E. coli* parent isolate had not significantly changed in this derivative (**Table 1**). Notably, whilst IncX3 plasmids have previously been seen to carry bla_{SHV-12} and bla_{NDM};[15] the bla_{NDM} gene located in our *K. pneumoniae* parent isolate did not co-transfer with bla_{SHV-12} into the *E. coli* parent isolate (**Table 1**) and it has been reported previously that bla_{SHV-12} has been identified on IncX3 plasmids lacking bla_{NDM} in *E. coli*.[16]

Whilst the *E. coli* parent (and resistant derivative) carry genes for bla_{TEM-1} and bla_{OXA-2}, only the former was detectably expressed. Whole genome sequencing confirmed that the reasons for this low-level expression are that the integron carrying bla_{OXA-2} is chromosomally located (so is single copy), bla_{OXA-2} is the third gene cassette in the integron (so is distant from the integron's common promoter); and the integron

promoter is of the weakest known designation.[17] Therefore, we conclude that the presence of three β-lactamases (CMY-4, CTX-M-15 and SHV-12) that all hydrolyse aztreonam [1] and that represent enzyme classes that are known to bind bicyclic boronate **2** [18,19] perhaps with a contribution from the resident TEM-1, which also binds the bicyclic boronate,[18] collectively has overcome utility of the inhibitor both by titration of the inhibitor and increased overall aztreonam hydrolysis. It is important to note, however, that the *ompF* porin gene is disrupted in our *E. coli* isolate as the result of an 8 bp insertion, leading to a frameshift and no detectable OmpF protein product (**Table 1**), and the role of reduced permeability to aztreonam or the inhibitor cannot be ruled out as a contributory factor.

The simple and fortuitous finding reported here has significant implications for the future of research into β-lactam/β-lactamase inhibitor resistance. It is known that β-lactamase hyperproduction – following gene duplication, promoter mutation, or mutations that stabilise the enzyme – can titrate out certain β-lactamase inhibitors in β-lactam/β-lactamase inhibitor combinations (e.g. amoxicillin/clavulanate or ceftazidime/avibactam), [3,20] but clearly another way of increasing the abundance of β-lactamase activity in a cell is to acquire an additional β-lactamase gene from a neighbouring bacterium, as we have found here. This could never be seen when testing individual isolates for their ability to generate resistant derivatives; either in the lab or using in vivo infection models. However, in the real world, whether during therapeutic use – at the site of infection in some cases, but certainly in the gut, for example – or in the environment if these chemicals are present for some reason, mixed populations of bacteria are found, increasing the potential for resistance to coalesce in one member of the population via horizontal gene transfer from the "β-lactamase-ome" of the population as a whole. Whilst this phenomenon of combined

mechanisms being necessary for resistance is not unique to β-lactamase inhibitors,[7] given their fixed concentration usage in MIC testing, it is likely to manifest itself more often due to titration effects. Another advantage of testing these mixed cultures for resistant derivatives is, therefore, that it can inform fixed concentration dosing of the inhibitor in the combination, to reduce the chance of resistance emerging in the clinic by the coalescence of resistance mechanisms found in a bacterial population.

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Transparency Declaration

None to declare – All authors.

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262 **Figure Legends**

263

264 **Figure 1: The accumulation of H33342 dye over a 30 cycle (4500 s) incubation**
265 **period by *K. pneumoniae* and *E. coli* isolates.** In each case, fluorescence of cells
266 incubated with the dye is presented as an absolute value after each cycle. Each line
267 shows mean data for three biological replicates with 8 technical replicates in each.
268 Error bars define the standard error of the mean.

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270

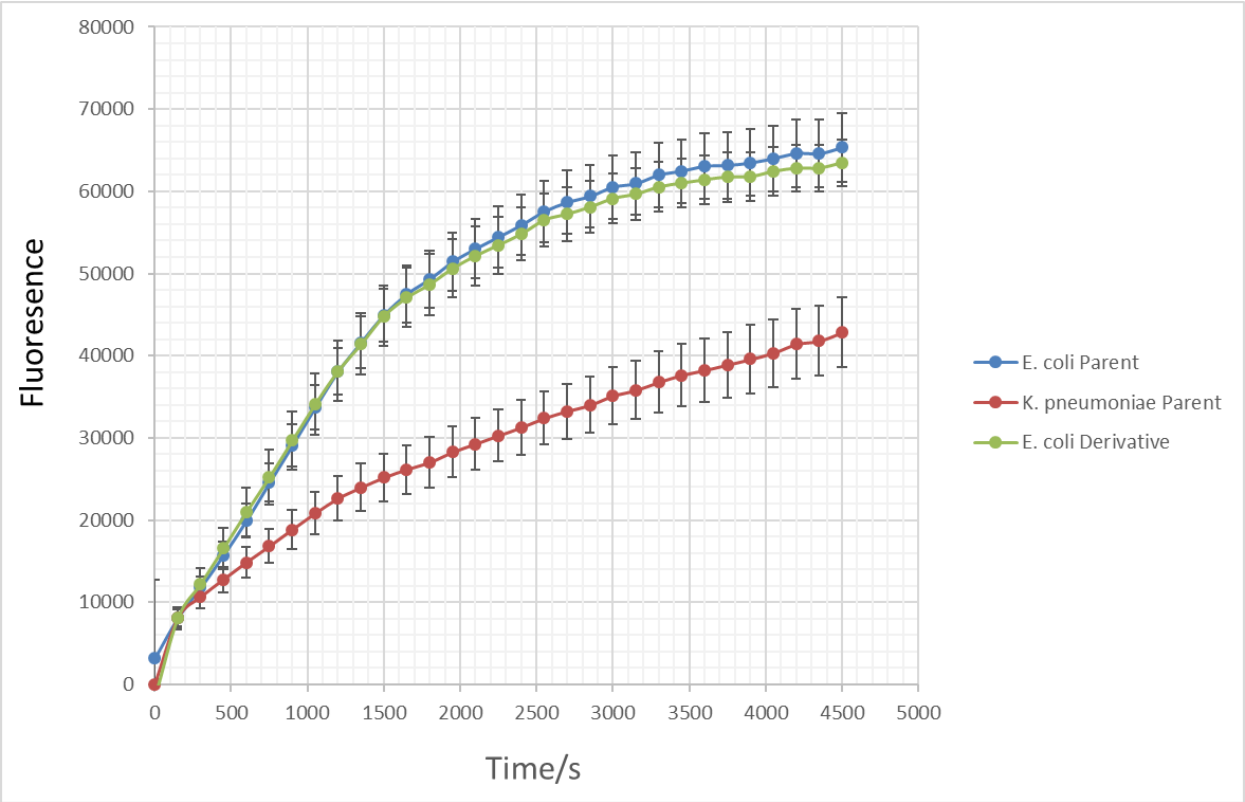
Tables

Table 1. Genotypic and Phenotypic Properties of *E. coli* and *K. pneumoniae* isolates.

Species and Sequence Type	Resistance gene complement (abundance of protein in proteome. Mean +/- SD normalised to average ribosomal protein, n=3)	Plasmid replicon complement
E. coli ST101 **(Parent)**	*aadA2, rmtB, strB, strA, armA, aac(3)-IIa, mph(E), msr(E), sul1, dfrA12, dfrA29, catA1* *bla*$_{CTX-M-15}$ (0.36 +/- 0.07) *bla*$_{TEM-1}$ (0.70 +/- 0.20) *bla*$_{OXA-2}$ (Not Detectable) *bla*$_{CMY-4}$ (0.85+/- 0.22) *ompF* (Not Detectable) *ompC* (4.48 +/- 0.78) *acrA* (0.43 +/- 0.07) *acrB* (0.14 +/- 0.06) *tolC* (0.20 +/- 0.11)	IncFII, IncA/C2, IncR IncAC[ST-1], IncF[F2:A-:B-]
K. pneumoniae ST625 **(Parent)**	*aadA2, aac(6')Ib-cr, aac(3)-IIa, strA, strB, rmtB, fosA, mph(A), sul2, sul1, dfrA12, aac(6')Ib-cr, qnrB7, qnrS1, tet(G), catB4, catA2* *bla*$_{SHV-12}$ *bla*$_{NDM-1}$ *bla*$_{CTX-M-15}$ *bla*$_{OKP-A-1}$ *bla*$_{OXA-1}$	IncX3, IncFII(pCRY), IncFIB(K), ColRNAI IncF[K-:A-:B-]
E. coli ST101 **(Aztreonam/ Boronate Resistant derivative)**	*aadA2, rmtB, strB, strA, armA, aac(3)-IIa, mph(E), msr(E), sul1, dfrA12, dfrA29, catA1* *bla*$_{SHV-12}$ (0.30 +/- 0.11) *bla*$_{CTX-M-15}$ (0.31 +/- 0.07) *bla*$_{TEM-1}$ (0.66 +/- 0.37) *bla*$_{OXA-2}$ (Not Detectable) *bla*$_{CMY-4}$ (0.70+/- 0.26) *ompF* (Not Detectable) *ompC* (2.95 +/- 1.63) *acrA* (0.21 +/- 0.02) *acrB* (0.11 +/- 0.04) *tolC* (0.12 +/- 0.08)	IncX3 IncFII, IncA/C2, IncR IncAC[ST-1], IncF[F2:A-:B-]

274 **Figure 1**

275



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