Selection of mutants with resistance or diminished susceptibility to ceftazidime/avibactam from ESBL- and AmpC- producing Enterobacteriaceae

David M LIVERMORE,[1,2]* Shazad MUSHTAQ,[1] Michel DOUMITH,[1] Dorota JAMROZY,[1,a] Wright W NICHOLS[3,b] and Neil WOODFORD[1]

[1]Antimicrobial Resistance and Healthcare Associated Infections (AMRHAI) Reference Unit, National Infection Service, Public Health England, 61 Colindale Avenue, LONDON NW9 5EQ; [2]Norwich Medical School, University of East Anglia, NORWICH NR4 7T; [3]Development Microbiology, AstraZeneca, Waltham MA02451, USA

Present addresses

[a]Wellcome Trust Sanger Institute, Hinxton, CB10 1SA

[b]Microbiology Consultant, Didsbury, Manchester, UK

***Corresponding author:**

Floor 2, Bob Champion Research & Educational Building,
James Watson Road, University of East Anglia,
Norwich Research Park, NORWICH, NR4 7UQ

Tel +44-(0)1603-597-568 or +44-(0)20-8327-7223

d.livermore@uea.ac.uk

Running head: CAZ-AVI-resistant mutants

Abstract

Introduction. Difficult Gram-negative infections are increasingly treated with new β-lactamase inhibitor combinations e.g. ceftazidime/avibactam. Disturbingly, mutations in KPC carbapenemases can confer ceftazidime/avibactam resistance, sometimes selected in therapy. We explored whether this risk extended to AmpC- and ESBL- enzymes. **Materials and Methods.** Mutants were selected by plating AmpC-derepressed strains, ESBL producers and ceftazidime-susceptible controls on agar containing ceftazidime + avibactam, 1 or 4 mg/L. MICs were determined by CLSI agar dilution; WGS was by Illumina methodology. **Results** Using 2x MIC of ceftazidime + 1 mg/L avibactam, mutants were selected from all strain types at frequencies of 10^{-7} - 10^{-9}. Rates diminished to $<10^{-9}$ with 4 mg/L avibactam or higher MIC multiples, except with AmpC-derepressed Enterobacteriaceae. Characterised mutants (n=10, MICs 4-64 mg/L) of AmpC-derepressed strains had modifications in *ampC*, giving Arg168Pro/HIs, Gly176Arg/Asp, Asn366Tyr or small deletions around positions 309-314. Mutants of ESBL producers (n=20; MICs 0.5-16 mg/L) mostly had changes affecting permeability, efflux or β-lactamase quantity; only one had an altered β-lactamase, with an Asp182Tyr substitution in CTX-M-15, raising the ceftazidime/avibactam MIC but abrogating other cephalosporin resistance. Mutants of ceftazidime-susceptible strains were not sequenced, but phenotypes suggested altered drug accumulation or, for *Enterobacter cloacae* only, AmpC derepression. In further experiments, avibactam reduced, but did not abolish, selection of AmpC-derepressed Enterobacteriaceae by ceftazidime. **Conclusions**. Most mutants of AmpC-derepressed Enterobacteriaceae had structural mutations in *ampC*; those of ESBL producers mostly had genetic modifications outside β-lactamase genes, commonly affecting uptake efflux or β-lactamase quantity. The clinical significance of these observations remains to be determined.

Introduction

Avibactam is the first diazabicyclooctane β-lactamase inhibitor to enter clinical use, formulated with ceftazidime and now licensed in both the US and the EU. An aztreonam/avibactam combination is in advanced development; development of a ceftaroline combination was pursued into Phase II but is now in abeyance.[1] Avibactam inhibits Class A β-lactamases, including ESBLs and KPC types, as well as Class C (AmpC) types.[2] Inhibitory activity against Class D β-lactamases is variable but, few of these are potent ceftazidimases. Metallo- (Class B) enzymes evade inhibition. As with all β-lactamase inhibitor combinations, activity also depends on the amount of β–lactamase, the underlying spectrum of the partner β-lactam and the permeability and efflux traits of the target strain.[3,4]

Single amino acid substitutions can reduce binding of clavulanate and penicillanic acid sulphones by TEM and SHV penicillinases,[5] but in-therapy selection of sequence variants of these enzymes is very rare.[6,7] Less is yet known on the potential of avibactam combinations to select resistance. In-vitro studies with ceftaroline/avibactam[8] yielded: (i) a single mutant of CTX-M-15 enzyme with a Lys237Gln substitution, and (ii) ceftaroline/avibactam-resistant mutants of AmpC-derepressed *Enterobacter* with deletions in the Ω-loop of AmpC, with Asn366His/Ile substitutions in AmpC or with porin modifications. The CTX-M-15 mutant conferred resistance to ceftaroline/avibactam but lost the ability of classical CTX-M-15 to cause resistance to other oxyimino-cephalosporins; the AmpC mutants were associated with broad resistance. We failed to select stable resistance to ceftaroline/avibactam in Enterobacteriaceae with other ESBLs besides CTX-M-15 or in those with KPC β–lactamase. [8] More recently, in-vitro and clinical selections of ceftazidime/avibactam-resistant mutants of Enterobacteriaceae with KPC carbapenemases have been described. Several mutations were seen in the laboratory mutants, mostly re-configuring the Ω-loop.[9] These alterations included Asp179Tyr, which has since been selected, during ceftazidime/avibactam therapy, in clinical mutants.[10] Its effect is to increase binding of ceftazidime,[11] protecting the KPC enzyme from inactivation by avibactam. Such

mutants show only small MIC rises for avibactam combinations other than ceftazidime/avibactam and often have reduced resistance to meropenem and aztreonam.[9,10] Clinical selection of reduced susceptibility, with the ceftazidime/avibactam MIC rising from 1 to 8 mg/L, was also described in a *Klebsiella pneumoniae* with OXA-48 and CTX-M-14. This was associated with Pro170Ser and Thr264Ileu mutations in the CTX-M-14 enzyme;[12] OXA-48, which lacks ceftazidimase activity, remained unchanged.

These observations led us to undertake selection studies with ceftazidime/avibactam, investigating a wide range of ESBLs and AmpC enzymes

Materials and Methods

Test strains

The test strains are detailed in Table 1 and were either reference organisms, isolates from survey collections.[13,14]. Work centred on *Escherichia coli, K. pneumoniae*, *Enterobacter cloacae* and *Citrobacter freundii*, as the major opportunistic Enterobacteriaceae and on TEM, SHV, CTX-M-15 and AmpC as the prevalent β–lactamases of concern.

β-Lactamase types were initially identified from phenotypes and PCR, but later confirmed by WGS. Controls for MIC testing comprised *E. coli* ATCC 25922, *E. coli* ATCC 35218 and *K. pneumoniae* ATCC 700603.

Antibiotics

Avibactam was provided by AstraZeneca (Wilmington, DE, USA), as were ceftazidime and ceftaroline; other antimicrobials were obtained from Sigma (Poole, UK), except ertapenem (Merck Sharp & Dohme, Hoddesdon, UK) and meropenem (AstraZeneca, Alderley Park, UK).

Single step mutant selection

Selection was undertaken as previously described for ceftaroline/avibactam.[8] Briefly *c.* 10^9 cfu from an overnight broth culture were spread on Mueller-Hinton agar (Thermofisher/Oxoid, Basingstoke, UK) containing ceftazidime/avibactam (fixed 1 or 4 mg/L concentration) at 2-16 x the MIC found previously by CLSI agar dilution. Colonies were counted after overnight incubation, and representatives retained for MIC determination and sequencing. Dilutions of the same overnight nutrient broth cultures were serially diluted and spread on to antibiotic-free Mueller-Hinton agar to provide a viable count, as a denominator for calculation of mutation frequencies.

Multi-step selection using β-lactamase producers

Inocula of 10^8 cfu were added to 10-mL amounts of nutrient broth containing ceftazidime/avibactam (with avibactam at 1 or 4 mg/L) at the ceftazidime/avibactam MICs found previously on agar with the same avibactam concentration, but otherwise by standard CLSI methodology,, and incubated up to 48 h.[8] This was repeated sequentially, each time doubling the ceftazidime concentration but keeping the avibactam concentration unchanged.

Selectivity for AmpC-derepressed mutants

Like other oxyimino-cephalosporins, ceftazidime can select AmpC-derepressed mutants from AmpC-inducible populations.[15] To test how avibactam might affect this phenomenon we plated *c.* 10^9 cfu of ceftazidime-susceptible (i.e., wild-type, AmpC-inducible, MIC $\leq$2 mg/L) cells of *E. cloacae* or *C. freundii* on to Mueller-Hinton agar with ceftazidime at 8 x MIC with or without 1 or 4 mg/L avibactam. After overnight incubation the colonies were counted and mutant frequencies calculated relative to the viable counts contained in the inocula.

MIC determinations

MICs were measured by CLSI agar dilution[16] for ceftazidime and for β-lactams in combination with β-lactamase inhibitors at the specified concentrations: ceftazidime/avibactam 1 and 4 mg/L, ceftazidime/cloxacillin 100 mg/L, ceftazidime/clavulanate 4 mg/L, ceftazidime/ tazobactam 4 mg/L, cefotaxime, cefepime, piperacillin/tazobactam 4 mg/L, ertapenem, meropenem, gentamicin and ciprofloxacin.

Genomic sequencing and bioinformatics analysis

Parent and mutant DNA were fragmented and tagged for multiplexing using the NexteraXT library preparation Kits (Illumina, Cambridge, UK) and sequenced on an Illumina HiSeq platform to produce 2x100 bp reads. Reads were assembled *de novo* using VelvetOptimiser software (http://www.vicbioinformatics.com/software.velvetoptimiser.shtml) with *k-mer* values from 55 to 75. Thepresence of β-lactamase variants was confirmed by BLAST searches, using the newly assembled genomes as query sequences against a reference database downloaded from the NCBI β-lactamase data (https://www.ncbi.nlm.nih.gov/pathogens/beta-lactamase-data-resources) resources.

Genomic alterations in mutants were identified as previously described.[17] sequencing reads for mutants were mapped to the de-novo assembled genome of the corresponding parent using Bowtie2 (http://bowtie-bio.sourceforge.net/bowtie2), and variants were called with Samtools with default paramters.[18] The variant calling files thereby generated were then parsed line by line to determine apparent alterations, with the accuracy of these predictions assessed based on read depth and mapping quality as described previously.[17] Sequences flanking confirmed alterations for 2-5 Kb on either side were extracted from the parent assemblies and manually inspected for gene structure and functional annotation with Blast searches so as to determine whether the detected changes were located in an open reading frame, promoter or intergenic region. Changes within structural genes were confirmed by aligning the protein-encoding sequences extracted from the parent and mutant assemblies. In high throughput sequencing, reads are randomly sampled, meaning that the number of

reads for any gene or gene fragment reflectsits copy number: We therefore counted the number of reads that mapped to the β-lactamase genes relative to those fo ther single-copy chromosomal *gyrA* and *parC* genes, thereby assessing whether the β-lactamase genes had been amplified in the mutants. Parent and mutant sequencing data are deposited in the European Nucleotide Archive under study number PRJEB27344 (www.ebi.ac.uk/ena).

Results

Mutant selection frequencies

Mutants were obtained from most strains, including β-lactamase-negative controls, when using ceftazidime/avibactam 1 mg/L at 2 x MIC, with frequencies of 10^{-7} to 10^{-9} (Table 1). Mutant frequencies were much reduced at higher MIC multiples or with 4 mg/L avibactam in the selective media. With 8- or 16-fold MIC multiples, mutants were obtained only from strains with stably derepressed AmpC; none was detected from ESBL producers or controls, even when the avibactam concentration was only 1 mg/L. Attempts to 'train' highly resistant mutants by multi-step procedures in broth were unsuccessful, with few mutants obtained; again mostly from strains with stably derepressed AmpC β-lactamases.

Mutants of AmpC derepressed strains

MICs were determined for 53 mutants selected from AmpC-derepressed *E. cloacae* or *C. freundii*. The MICs of ceftazidime/avibactam 4 mg/L rose from 0.5-2 mg/L for the parent strains to 4-64 (mostly 8-16) mg/L for the mutants; in 23/53 cases the values for the mutants were >8+4 mg/L, exceeding the CLSI/EUCAST breakpoint. MICs of ceftazidime combined with cloxacillin 100 mg/L (which inhibits AmpC) also were widely, though not universally, raised: MICs of ceftaroline/avibactam rose little, generally only from 0.25-2 mg/L to 1-2 mg/L. Shifts in the MICs of other β–lactams were erratic: some mutants showed rises in cefotaxime

and cefepime MICs whereas others showed falls. Likewise, a few mutants showed increases in ertapenem MIC, but falls were commoner.

This diversity is illustrated in Table 2 for the 10 mutants of AmpC-derepressed *E. cloacae and C. freundii* selected for WGS. All proved to have modifications in *ampC,* resulting in amino acid substitutions, including Arg168Pro (three representatives), Arg168His (two representatives), Gly176Arg/Asp (one representative each) Asn366Tyr (one representative) or two- to four- amino acid deletions around positions 309-314 (two representatives). The Arg168Pro substitution was associated with reduced resistance to all cephalosporins, in both *E. cloacae* and *C. freundii* along with the complete loss of synergy between avibactam and both ceftazidime and ceftaroline, whereas Arg168His and Gly176Arg/Asp were associated with retention of broad cephalosporin resistance and ceftaroline/avibactam synergy coupled with markedly reduced ceftazidime/avibactam synergy.

Characterisation of selected mutants: ESBL producers

Among 63 mutants of nine ESBL producers (the tenth failed to yield any mutants), only 12 achieved resistance to ceftazidime/avibactam as defined by CLSI, with MICs of 16+4 or 32+4 mg/L. All these 12 were selected from the same parent, *E. coli* J53-1 with TEM-10 ceftazidimase. The ceftazidime/avibactam MICs for other mutants were raised, but with values $\leq$8+4 mg/L. MICs for 20 mutants selected to represent phenotype diversity are shown in Table 3, along with details of the genetic modifications revealed by WGS.

Most mutants of ESBL producers had sequence changes in genes related to permeability, efflux or β-lactamase expression, not in β-lactamase coding genes. Thus, 7/20 had modifications in *ompR/envZ*, which regulates expression of porins OmpC and OmpF;[20] 2/20 had identical alterations in *acrAB* efflux gene components; and 9/20 either yielded increased reads of β-lactamase genes relative to *gyrA* and *parC* during WGS, implying gene

amplification, or had sequence changes upstream of β-lactamase genes that putatively might increase their expression, though this was not investigated by experiment. Almost all these mutants of ESBL producers showed broad upward rises for β-lactam MICs, including other inhibitor combinations besides ceftazidime/avibactam. A representative ('Mutant 5') of the group of *E. coli* J53-1 TEM-10 mutants with ceftazidime/avibactam MICs of 16+4 mg/L (i.e. the most-resistant mutants selected from ESBL producers, see above) had changes in both *envZ* and upstream of bla_{TEM}; MICs of 4+4 mg/L were recorded for Mutants 2, 3 and 4 of the same parent, and these only had the lesion upstream of bla_{TEM}, not that in *envZ*.

Just one of the 20 mutants sequenced – *E. coli* EO 553 Mutant 3 – had a lesion in its ESBL-encoding gene, leading to an Asp182Tyr substitution in CTX-M-15. Compared with its parent, this mutant lost resistance to ceftaroline, cefotaxime and cefepime, and the ceftazidime MIC was reduced two-fold from 32 to 16 mg/L. Synergy was completely lost between ceftazidime and clavulanate or tazobactam whilst the ceftazidime/avibactam MIC rose 8-fold, from 0.25 mg/L to 2 mg/L.

One further mutant – Mutant 8 of *K. pneumoniae* Mei 838 – had a lesion in *mdrA*, which encodes penicillin-binding protein 2. This may act as a secondary target for diazabicyclooctanes, though this effect is much weaker for avibactam than for the developmental analogues nacubactam and zidebactam.[21,22] The significance of this lesion is difficult to judge.

Finally, several mutants had changes in proteins with no obvious link to β-lactam or diazabicyclooctane action, including (i) aspartate semialdehyde dehydrogenase (Mutant 7 of *K. pneumoniae* Mei 838), (ii) 4-cytidine 5-diphospho-2-C-methyl D erythritol kinase and putative sulphate transporter (both in Mutant 8 of *K. pneumoniae* Mei 838) and (iii) the DNA-binding protein HLP-II pleiotropic regulator (Mutant 14 of *K. pneumoniae* Mei 254).

Mutants from control Enterobacteriaceae lacking ceftazidime resistance

Mutants of ceftazidime-susceptible *E. coli* and *K. pneumoniae* were obtained under selection with ceftazidime/avibactam 1 mg/L, though not ceftazidime/avibactam 4 mg/L (Table 1). MICs of ceftazidime/avibactam 1 mg/L rose from 0.06 to 0.25 mg/L for the parent strains to 0.5-4 mg/L for the mutants, whilst those of ceftazidime/avibactam 4 mg/L rose from 0.015-0.25 mg/L to 0.12-2 mg/L. These shifts were accompanied by small, generalised, rises in the MICs of other β-lactams and inhibitor combinations and – often – ciprofloxacin. Given this spectrum, permeability or efflux mechanisms are likely, and these were not pursued further.

Similar small but broad MICs shifts were seen for 2/12 characterised mutants of the AmpC-inducible *Enterobacter* strain LN07013 selected with low MIC multiples of ceftazidime/avibactam 1 mg/L. However, 10/12 mutants had antibiograms suggesting AmpC-derepression, with high-level resistance to ceftazidime, cefotaxime, ceftaroline and piperacillin/tazobactam (MICs rising from $\leq$1 mg/L to $\geq$128 mg/L) but not to cefepime or carbapenems. Despite selection with ceftazidime/avibactam, ceftazidime resistance continued to be largely reversed by avibactam, with the MIC falling from $\geq$128 mg/L for the unprotected cephalosporin to 2 mg/L with 1 mg/L avibactam and to 0.5-2 mg/L with 4 mg/L avibactam or to 1-8 mg/L with 100 mg/L cloxacillin.

These observations led us to investigate the effect of avibactam on the selectivity of ceftazidime for AmpC-derepressed mutants from AmpC-inducible population of *E. cloacae* and *C. freundii*. We plated five AmpC-inducible, ceftazidime-susceptible strains of each of these species on to agar with ceftazidime alone or with avibactam at 1 or 4 mg/L, always at 8 x MIC. Large numbers of colonies were recovered on plates containing ceftazidime alone, indicating mutation frequencies of 10^{-6} to 10^{-7}, as is typical for these species.[23] With avibactam at 1 mg/L, the numbers of colonies that grew were reduced by >75% in all cases and, for 8/10 strains, fell below the detection limit of 10^{-9}; with avibactam at 4 mg/L, the mutant frequency fell below 10^{-9} for 9/10 strains.

Discussion

Mutational resistance to ceftazidime/avibactam has become a concern in respect of isolates with KPC carbapenemases, with reports of resistance emerging during therapy as well as *in vitro*.[9,10] In the light of this concern we explored whether ceftazidime/avibactam could also select resistant mutants from AmpC-derepressed Enterobacteriaceae and ESBL producers; the work followed a similar previous study for ceftaroline/avibactam.[8] We included common and representative ESBLs, specifically CTX-M-15, as the most prevalent type; CTX-M-1, as common from animal isolates; SHV-2 and -5 as major ESBL mutants of SHV-1 and TEM-10 as a ceftazidimase-type ESBL. *E. cloacae* and *C. freundii* were prioritsed as the major species where high-level expression AmpC is a resistance issue. Clearly there are further enzymes that merit investigation in the future, notably including Group 9 CTX-M- types (CTX-M-9 or -14), which are globally frequent, and broad-spectrum TEM ESBLs (e.g. CTX-M-3).

Unlike for KPC carbapenemases, where we found much higher mutation frequencies to ceftazidime/avibactam than to ceftaroline/avibactam,[8,9] the present data substantially mirror those obtained with ceftaroline/avibactam, with (i) low mutation frequencies ($<10^{-8}$ at above 2 x MIC), particularly when selection was done with 4 mg/L avibactam, (ii) with mostly small rises in ceftazidime/avibactam MICs, rather than frank resistance and (iii) with most emerging resistance seen among AmpC derepressed strains rather than those with ESBLs.

Critically, and also in keeping with the previous ceftaroline/avibactam work, we found that the mutants of AmpC derepressed strains typically had changes within *ampC* whereas mutants derived from ESBL producers largely had mutations affecting efflux, permeability or, putatively, β-lactamase quantity. Nevertheless, there were differences: hot spots for mutations associated here with ceftazidime/avibactam non-susceptibility in AmpC hyperproducers were amino acids 168, 176, 309-314 and 366; those conferring ceftaroline/avibactam resistance were around the Ω loop (amino acids 213-226) or, again, residue 366. Mutations at Arg168 – the site most often affected in this study – had differing

effects: Arg168Pro reduced resistance to all cephalosporins and abrogated synergy between avibactam and both ceftazidime and ceftaroline. This behaviour would be compatible with loss of affinity for avibactam, though this was not investigated biochemically. On the other hand, Arg168His (and Gly176Arg/Asp) raised ceftazidime/avibactam MICs but had little effect on cephalosporin resistance overall or on synergy between ceftaroline and avibactam – behaviours that are more compatible with the mutation conferring increased affinity for ceftazidime. The substitutions were all at conserved positions, with a caveat that the background variation among the primary sequences of AmpC β-lactamases from *E. cloacae* was about 40% in one study.[24] Asn366 (designated as Asn346 after discounting the 20-amino acid signal peptide) has previously been described as a key residue for avibactam binding.[24] The deletions around positions 309–314 observed here are in helix H10, close to the enzyme active site, and at the location where a 6-amino-acid deletion in clinical isolate *E. cloacae* CHE was associated with expansion of the enzyme's activity and diminished susceptibility to avibactam combinations.[19].

The sole mutant of an ESBL enzyme selected here was an Asp185Tyr variant of CTX-M-15. This change was associated with the reduction or loss of resistance to other cephalosporins besides ceftazidime. It seems unlikely that a mutant with such a narrowed resistance spectrum would be successful in evolutionary terms, implying little public health risk. Similar points were made previously in respect of a Lys237Gln mutant selected with ceftaroline/avibactam:[8] compared with its parent organism this gained ceftaroline/avibactam resistance but lost resistance to other oxyimino-cephalosporins, including ceftazidime. The other mutants selected in the present study from ESBL producers largely had efflux or permeability modifications, or had mutations and amplifications suggesting increased β–lactamase expression - a known general correlate with reduced susceptibility to β-lactam/β–lactamase inhibitor combinations.[4]

Mutants selected from cephalosporin-susceptible AmpC and ESBL-negative *E. coli* and *K. pneumoniae* only showed small increases in ceftazidime/avibactam MICs and were

not characterised in detail. Again, however, their antibiograms suggested permeation- or efflux-related changes. By contrast, most of the *E. cloacae* mutants had antibiograms suggesting AmpC derepression. Although avibactam inhibits this enzyme, MICs of ceftazidime/avibactam for AmpC derepressed organisms are not quite so low as for inducible ones and there appears to be a small concentration window (as e.g. with cefepime)[25] in which derepressed mutants may be selected, though these remain susceptible to ceftazidime/avibactam at breakpoint in the absence further changes to *ampC* itself

Only clinical experience will show whether the present observations have clinical significance. Overall, they suggest that the potential for emerging resistance ceftazidime/avibactam is greater with AmpC producers than ESBL producers. Thus far we are unaware of any reports of emerging resistance during clinical use against AmpC producers. There is a single report[12] of emerging resistance in a pneumonia patient, with a *K. pneumoniae* producing OXA-48 carbapenemase together with CTX-M-14, an ESBL not studied here. The patient was treated first with ceftazidime plus colistin and later, after the ceftazidime MIC had risen from 4 to >256 mg/L, with ceftazidime/avibactam plus meropenem. During this latter phase of therapy the ceftazidime/avibactam MIC rose from 1 to 8 mg/L and the CTX-M-14 enzyme acquired Pro170Ser and Thr264Ile substitutions, whilst the OXA-48 carbapenemase remained unaltered. This one case must, however, be set against the clinical trials, where ESBL producers were well represented, without emerging resistance;[26-28] these support our view that the selection risk with ESBL producers is low whereas that with AmpC-derepressed organisms will only be clarified by clinical experience.

Acknowledgement: We are grateful to Dr S Lahiri for helpful discussions on structure activity relationships in AmpC enzymes

Funding: This work was funded by AstraZeneca prior to transfer of their antibiotic portfolio, including ceftazidime/avibactam, to Pfizer.

Transparency declaration

DML: Advisory Boards or ad-hoc consultancy for Accelerate, Achaogen, Adenium, Allecra, AstraZeneca, Auspherix, Basilea, BioVersys, Centauri, Discuva, Meiji, Nordic, Pfizer, Roche, Shionogi, T.A.Z., Tetraphase, The Medicines Company, VenatoRx, Wockhardt, Zambon, Zealand. Paid lectures – Astellas, AstraZeneca, bioMérieux, Beckmann Coulter, Cardiome, Cepheid, Merck, Pfizer and Nordic. Relevant shareholdings in– Dechra, GSK, Merck, Perkin Elmer, Pfizer amounting to <10% of portfolio value. **WWN:** At the time of the study, WWN was an employee of AstraZeneca and owns shares in that company. He has also been a paid consultant for Pfizer. **All other authors:** none to declare. However, PHE's AMRHAI Reference Unit has received financial support for conference attendance, lectures, research projects or contracted evaluations from numerous sources, including: Accelerate Diagnostics, Achaogen Inc, Allecra Therapeutics, Amplex, AstraZeneca UK Ltd, AusDiagnostics, Basilea Pharmaceutica, Becton Dickinson Diagnostics, bioMérieux, Bio-Rad Laboratories, The BSAC, Cepheid, Check-Points B.V., Cubist Pharmaceuticals, Department of Health, Enigma Diagnostics, Food Standards Agency, GlaxoSmithKline Services Ltd, Helperby Therapeutics, Henry Stewart Talks, IHMA Ltd, Innovate UK, Kalidex Pharmaceuticals, Melinta Therapeutics, Merck Sharpe & Dohme Corp, Meiji Seika Pharma Co., Ltd, Mobidiag, Momentum Biosciences Ltd, Neem Biotech, Nordic Pharma Ltd, Norgine Pharmaceuticals, Rempex Pharmaceuticals Ltd, Roche, Rokitan Ltd, Smith & Nephew UK Ltd, Shionogi & Co. Ltd, Trius Therapeutics, VenatoRx Pharmaceuticals and Wockhardt Ltd.

References

1. Bush K. A resurgence of β-lactamase inhibitor combinations effective against multidrug-resistant Gram-negative pathogens. *Int J Antimicrob Agents* 2015; **46:** 483-93.
2. Ehmann DE, Jahic H, Ross PL *et al.* Kinetics of avibactam inhibition against Class A, C, and D β-lactamases. *J Biol Chem* 2013; **288:** 27960-71.

3. Livermore DM. Determinants of the activity of β-lactamase inhibitor combinations. *J Antimicrob Chemother* 1993; **31 Suppl A:** 9-21.

4. Livermore DM, Meunier D, Hopkins KL *et al.* Activity of ceftazidime/avibactam against problem Enterobacteriaceae and *Pseudomonas aeruginosa* in the UK, 2015-16. *J Antimicrob Chemother* 2017 Dec 8. doi: 10.1093/jac/dkx438.

5. Cantón R, Morosini MI, de la Maza OM *et al.* IRT and CMT β-lactamases and inhibitor resistance. *Clin Microbiol Infect* 2008; **14 Suppl 1:** 53-62.

6. Claeys G, De Baere T, Vaneechoutte M *et al.* Caz-hi, and extended-spectrum TEM β-lactamase (TEM-61), is derived from Caz-lo (TEM-11) by in vivo selection. Antimicrob Agents Chemother 1998; **42:** 3328-9.

7. Jacquier H, Marcadé G, Raffoux E *et al.* In vivo selection of a complex mutant TEM (CMT) from an inhibitor-resistant TEM (IRT) during ceftazidime therapy. *J Antimicrob Chemother* 2013; **68:** 2792-6.

8. Livermore DM, Mushtaq S, Barker K *et al.* Characterization of β-lactamase and porin mutants of Enterobacteriaceae selected with ceftaroline + avibactam (NXL104). J Antimicrob Chemother. 2012; **67:** 1354-8.

9. Livermore DM, Warner M, Jamrozy D *et al.* In vitro selection of ceftazidime-avibactam resistance in Enterobacteriaceae with KPC-3 carbapenemase. *Antimicrob Agents Chemother* 2015; **59:** 5324-30.

10. Shields RK, Potoski BA, Haidar G *et al.* Clinical outcomes, drug toxicity, and emergence of ceftazidime-avibactam resistance among patients treated for carbapenem-resistant Enterobacteriaceae infections. *Clin Infect Dis* 2016; **63:** 1615-8.

11. Winkler ML, Papp-Wallace KM, Bonomo RA. Activity of ceftazidime/avibactam against isogenic strains of *Escherichia coli* containing KPC and SHV β-lactamases with single amino acid substitutions in the Ω-loop. *J Antimicrob Chemother* 2015; **70:** 2279-86.

12. Both A, Büttner H, Huang J *et al.* Emergence of ceftazidime/avibactam non-susceptibility in an MDR Klebsiella pneumoniae isolate. *J Antimicrob Chemother* 2017; **72:** 2483-8.

13. Livermore DM, Yuan M. Antibiotic resistance and production of extended-spectrum β-lactamases amongst *Klebsiella* spp. from intensive care units in Europe. *J Antimicrob Chemother* 1996; **38:** 409-24.

14. Potz NA, Hope R, Warner M *et al.* Prevalence and mechanisms of cephalosporin resistance in Enterobacteriaceae in London and South-East England. *J Antimicrob Chemother* 2006; **58:** 320-6.

15. Negri MC, Baquero F. In vitro selective concentrations of cefepime and ceftazidime for AmpC beta-lactamase hyperproducer *Enterobacter cloacae* variants. *Clin Microbiol Infect* 1999; **5 Suppl 1:** S25-8.

16. Clinical and Laboratory Standards Institute. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically, Tenth Edition: Approved Standard M7-A10.* CLSI, Wayne, PA, USA, 2015.

17. Doumith M, Mushtaq S, Livermore DM *et al.* New insights into the regulatory pathways associated with the activation of the stringent response inbacterial

resistance to the PBP2-targeted antibiotics, mecillinam andOP0595/RG6080. *J Antimicrob Chemother* 2016; **71:** 2810-4.

18. Li H, Handsaker B, Wysoker A, Fennell T *et al.* The sequence alignment/map format and SAMtools. *Bioinformatics* 2009; **25:** 2078-9.

19. Lahiri SD, Giacobbe RA, Johnstone M *et al.* Activity of avibactam against *Enterobacter cloacae* producing an extended-spectrum class C β-lactamase enzyme. *J Antimicrob Chemother* 2014; **69:** 2942–6

20. Mizuno T, Mizushima S. Signal transduction and gene regulation through the phosphorylation of two regulatory components: the molecular basis for the osmotic regulation of the porin genes. *Mol Microbiol* 1990; **4:** 1077-82.

21. Morinaka A, Tsutsumi Y, Yamada M *et al.* OP0595, a new diazabicyclooctane: mode of action as a serine β-lactamase inhibitor, antibiotic and β-lactam 'enhancer'. *J Antimicrob Chemother* 2015; **70:** 2779-86.

22. Livermore DM, Mushtaq S, Warner M *et al.* In vitro activity of cefepime/zidebactam (WCK 5222) against Gram-negative bacteria. *J Antimicrob Chemother* 2017; **72:** 1373-85.

23. Livermore DM. Clinical significance of β-lactamase induction and stable derepression in Gram-negative rods. *Eur J Clin Microbiol* 1987; **6:** 439-45.

24. Lahiri SD, Johnstone MR, Ross PL *et al.* Avibactam and class C β-lactamases: mechanism of inhibition, conservation of the binding pocket, and implications for resistance. *Antimicrob Agents Chemother* 2014; **58:** 5704-13.

25. Piddock LJ, Griggs DJ. Selection and characterization of cefepime-resistant Gram-negative bacteria. *J Antimicrob Chemother* 1991; **28:** 669-76.

26. Mendes RE, Castanheira M, Gasink L *et al.* β-Lactamase characterization of Gram-negative pathogens recovered from patients enrolled in the Phase 2 trials for ceftazidime-avibactam: clinical efficacies analyzed against subsets of molecularly characterized isolates. *Antimicrob Agents Chemother* 2015; **60:** 1328-35.

27. Mendes RE, Castanheira M, Woosley LN *et al.* Molecular β-lactamase characterization of aerobic Gram-negative pathogens recovered from patients enrolled in the ceftazidime-avibactam Phase 3 trials for complicated intra-abdominal infections, with efficacies analyzed against susceptible and resistant subsets. *Antimicrob Agents Chemother* 2017; **61:** pii: e02447-16.

28. Carmeli Y, Armstrong J, Laud PJ *et al.* Ceftazidime-avibactam or best available therapy in patients with ceftazidime-resistant Enterobacteriaceae and *Pseudomonas aeruginosa* complicated urinary tract infections or complicated intra-abdominal infections (REPRISE): a randomised, pathogen-directed, phase 3 study. *Lancet Infect Dis* 2016; **16:** 661-673.

452 **Table 1.** Frequencies of single step mutants selected on agar containing MIC multiples of ceftazidime/avibactam

Isolate No.	Species	Mech-anism	Parent MIC (mg/L)			Initial count X 10^9	Selection frequency x 10^{-9} using							
							CAZ-AVI 1 at MIC multiple				CAZ-AVI 4 at MIC multiple			
			CAZ	CAZ-AVI 1	CAZ-AVI 4		2 x	4 X	8 X	16 X	2 x	4 X	8 X	16 X
Mei 633	*K. pneumoniae*	SHV-2	>256	4	1	1.11	<	<	<	<	1.8	<	<	<
Mei 838	*K. pneumoniae*	SHV-2	64	4	0.5	1.23	8.9	<	<	<	3.3	<	<	<
Mei 254	*K. pneumoniae*	SHV-5	256	4	0.5	1.10	57.4	<	<	<	1020.0	16.4	<	<
Mei 679	*K. pneumoniae*	SHV-5	>256	4	1	1.63	<	<	<	<	<	<	<	<
LN01001	*K. pneumoniae*	CTX-M-1	64	0.5	0.5	1.46	2.7	<	<	<	<	<	<	<
LN01028	*K. pneumoniae*	CTX-M-1	256	2	0.5	1.41	2.9	<	<	<	<	<	<	<
EO 553	*E. coli*	CTX-M-15	16	0.5	0.25	1.27	3.2	<	<	<	0.8	<	<	<
EO 499	*E. coli*	CTX-M-15	32	0.25	0.25	0.92	1.1	<	<	<	<	<	<	<
NCTC13352	*E. coli*	TEM-10	>256	2	0.5	1.04	183.9	<	<	<	28.7	<	<	<
J53 TEM-10	*E. coli*	TEM-10	>256	2	1	1.22	26.3	1.7	<	<	18.9	6.6	<	<
LN03019	*E. cloacae*	AmpC SDR	64	1	0.5	1.51	26.6	<	<	<	<	<	<	<
LN07047	*E. cloacae*	AmpC SDR	64	1	0.5	1.04	835.6	7.7	1.0	1.9	21.2	4.8	1.9	<
LN10061	*C. freundii*	AmpC SDR	256	2	1	1.13	<	<	<	<	<	<	<	<
SE01073	*C. freundii*	AmpC SDR	128	1	0.5	0.91	25.3	7.7	3.3	<	8.8	2.2	2.2	<
LN01QC09	*E. coli*	CAZ S	0.125	0.125	0.125	1.10	Cont.	<	<	<	<	<	<	<
LN04QC03	*E. coli*	CAZ S	0.125	0.125	0.125	1.01	Cont.	<	<	<	13.9	<	<	<
LN07013	*E. cloacae*	CAZ S	0.5	0.25	0.25	1.60	267.8	14.4	<	<	1.3	<	<	<

LN09063A	*E. cloacae*	CAZ S	0.25	0.25	0.25	1.63	0.6	<	<	<	<	<	<	<
Mei 60	*K. pneumoniae*	CAZ S	0.12	0.12	0.12	1.14	3.5	<	<	<	<	<	<	<
Mei 888	*K. pneumoniae*	CAZ S	0.12	0.12	0.25	1.40	982.8	22.9	<	<	0.7	<	<	<

<: Below detection limit of c. 0.5 $x10^{-9}$

Abbreviations: CAZ, ceftazidime, CAZ-AVI1 ceftazidime with 1 mg/L avibactam; CAZ-AVI4 ceftazidime with 4 mg/L avibactam; Cont: contaminated; CAZ-S ceftazidime-susceptible; SDR, stably derepressed

458 **Table 2.** Characterisation of mutants selected from AmpC derepressed *E. cloacae* and *C. freundii*

Strain/mutant and selection conditions	AmpC mutation[a]	Porin status	Other	MIC (mg/L)														
				Ceftazidime						Ceftaroline		Others						
				Alone	+Avi, 1 mg/L	+Avi, 4 mg/L	+Clox 100 mg/L	+Clav 4 mg/L	+Taz 4 mg/L	Alone	+Avi, 4 mg/L	CTX	CPM	PTZ	MEM	ERP	GEN	CIP
***E. cloacae* LN07047**		**OmpC/F functional**		**128**	**1**	**0.5**	**2**	**128**	**64**	**>32**	**0.5**	**256**	**0.5**	**64**	**0.06**	**0.5**	**0.5**	**0.015**
Mutant 4 (CAZ2-AVI 1)	Gly176Arg	Unchanged		>256	256	64	>256	>256	>256	>32	2	>256	2	16	0.03	0.25	0.5	0.015
Mutant 7 (CAZ4-AVI1)	Gly176Asp	Unchanged		128	32	16	128	256	128	>32	2	64	0.125	8	0.03	0.06	0.5	0.015
Mutant 15 (CAZ1-AVI4)	Deletion Leu313, Ala314	Unchanged	OppB oligopeptide/ nickel transporter Tyr272Asp	256	16	8	8	256	128	16	1	16	16	32	0.03	0.125	0.5	0.015
Mutant 19 (CAZ2-AVI4)	Arg168Pro	Unchanged		16	16	16	16	64	32	4	2	2	2	8	0.03	0.03	0.5	0.015
Mutant 24 (CAZ4-AVI4)	Arg168Pro	Unchanged		16	16	16	16	32	32	2	2	0.5	2	8	0.03	0.03	0.5	0.03
***C. freundii* SE01073**		**OmpF inactivated (IS)**		**128**	**1**	**0.5**	**2**	**128**	**64**	**32**	**0.125**	**32**	**1**	**64**	**0.06**	**0.25**	**0.5**	**0.015**
Mutant 5 (CAZ2-AVI1)	Arg168Pro	Unchanged		16	32	8	32	32	32	0.5	0.25	2	4	16	0.015	0.015	1	0.008
Mutant 8 (CAZ4-AVI)	Deletion309-312 Ser-Lys-Val-Ala; Leu313Met	Unchanged		256	32	8	128	256	256	8	0.25	8	4	64	0.03	0.03	1	0.008
Mutant 9 (CAZ4-AVI1)	Arg168His	Unchanged		256	32	4	128	256	256	32	0.25	32	16	64	0.03	0.06	1	0.008

Mutant 12 (CAZ8-AVI1)	Asn366Tyr	Unchanged	DnaK Molecular chaperone Leu273Gln	128	64	16	64	128	128	16	2	16	4	64	0.03	0.03	1	0.03
Mutant 22 (CAZ4-AVI4)	Arg168His	Unchanged	Aldehyde dehydrogenase Arg407His	128	64	32	64	256	128	16	2	16	4	64	0.03	0.03	1	0.015

459

460 Parent strains are shown in bold font

461 [a] Numbering here is from the first amino acid of the coding sequence as in figure 3 of ref [19]. The first 20 amino acids comprise a signal peptide, cleaved from the mature
462 protein and are discounted in some numberings

463 **Table 3.** Characterisation of mutants selected from ESBL producers

					MICs (mg/L)														
Strain and selective conditions	β-lactamase(s)	Porin status[a]	Efflux[a]	Other[a]	Ceftazidime						Ceftaroline		others						
					Alone	+Avi, 1 mg/L	+Avi, 4 mg/L	+Clox 100 mg/L	+Clav 4 mg/L	+Taz 4 mg/L	Alone	+Avi, 4 mg/L	CTX	CPM	PTZ	MEM	ERP	GEN	CIP
***E. coli* EO 499**	**CTX-M-15, OXA-1. TEM-1**	**OmpC OmpF both active**			**32**	**0.25**	**0.125**	**32**	**0.25**	**0.5**	**>32**	**0.03**	**256**	**16**	**16**	**0.03**	**0.06**	**1**	**>16**
Mutant 1 CAZ1-AVI1[b]	*bla*$_{CTX-M-15}$ up from *c.* 5 to 40 copies; *bla*$_{TEM}$/*bla*$_{OXA}$ unchanged				>256	2	0.5	>256	2	32	>32	0.06	>256	>64	>256	0.125	0.5	1	>16
***E. coli* EO 553**		**OmpC OmpF both active**			**32**	**0.5**	**0.25**	**32**	**1**	**1**	**>32**	**0.06**	**256**	**32**	**4**	**0.03**	**0.06**	**0.5**	**>16**
Mutant 2 CAZ1-AVI1			AcrB Phe615Ser		64	2	2	64	2	4	>32	0.125	>256	64	0.5	0.03	0.06	1	>16
Mutant 3 CAZ1-AVI1	CTX-M-15 Asp182Tyr				16	8	2	16	8	16	0.5	0.125	0.5	0.25	4	0.03	0.03	1	>16
Mutant 5 CAZ0.5-AVI4		EnvZ : Val132Gly	AcrB Phe615Ser		64	4	4	64	4	4	>32	0.5	>256	64	0.5	0.06	0.06	0.5	>16
***K. pneumoniae* Mei 838**	**SHV-2**	**OmpK35, OmpK36 both active**			**64**	**2**	**0.25**	**64**	**1**	**32**	**>32**	**0.06**	**64**	**16**	**>256**	**0.03**	**0.125**	**0.5**	**0.03**
Mutant 6 CAZ1-AVI4	*bla*$_{SHV-2}$ up from *c.* 15 to 70 copies	OmpR: Arg15His			>256	32	8	>256	8	>256	>32	0.5	>256	>64	>256	0.06	1	1	0.06
Mutant 7 CAZ1-AVI4	*bla*$_{SHV-2}$ up from *c.* 15 to 45 copies			Aspartate-semialde-hyde de-hydrogen-ase	>256	32	2	>256	8	>256	>32	0.5	>256	>64	>256	0.06	1	1	0.03

				Gln247Leu														
Mutant 8 CAZ1-AVI4				4-Cytidine 5'-diphospho-2-C-methyl-D-erythritol kinase Ala270Gly Putative sulphate transporter (*ychM*) Ala99Gly	>256	32	2	>256	8	>256	>32	0.5	256	>64	>256	0.06	0.5	1	0.06
Mutant 9 CAZ1-AVI4				Penicillin binding protein 2 *mdrA*: Asp354Ala	>256	16	8	>256	8	256	>32	0.5	>256	>64	>256	0.06	0.5	1	0.06
***K. pneumoniae* LN01028**	**CTX-M-15**	**OmpK35, OmpK36 both active**			**256**	**1**	**0.5**	**256**	**16**	**8**	**>32**	**0.125**	**256**	**64**	**32**	**0.125**	**2**	**>32**	**2**
Mutant 1 CAZ4-AVI1		EnvZ : Arg397Cys			>256	16	4	>256	4	>256	>32	0.125	>256	64	>256	0.06	0.5	>32	4
Mutant 2 CAZ4-AVI1				Peptido-glycan-associated outer membrane lipo-protein Met1Ileu	>256	16	4	>256	8	>256	>32	0.25	>256	>64	>256	0.06	0.5	>32	4
Mutant 4 CAZ4-AVI1		EnvZ : Ileu412Leu			>256	16	4	>256	4	>256	>32	0.125	>256	>64	>256	0.06	0.5	>32	4
***K. pneumoniae* Mei 254**	**SHV-5**	**OmpK35, OmpK36 both active**			**256**	**1**	**0.25**	**256**	**0.5**	**2**	**8**	**0.125**	**16**	**2**	**8**	**0.03**	**0.06**	**0.5**	**1**

Mutant 7 CAZ1-AVI4					>256	32	8	>256	8	>256	>32	0.25	256	>64	>256	0.125	1	0.5	1
Mutant 14 CAZ2-AVI4				DNA-binding protein HLP-II pleiotropic regulator Ser2Arg	>256	16	4	>256	4	>256	>32	0.25	128	32	>256	0.125	1	0.5	1
***E. coli* NCTC 13352**	**TEM-10**	**OmpC, OmpF both active**			**>256**	**1**	**0.5**	**256**	**0.5**	**2**	**>32**	**0.06**	**1**	**1**	**4**	**0.03**	**0.03**	**0.5**	**0.015**
Mutant 9 CAZ1-AVI4	*bla*$_{TEM}$ copy number up from 80 to 200	EnvZ Gln115Arg			>256	16	8	>256	8	>256	>32	0.25	32	>64	>256	0.125	1	1	0.03
Mutant 10 CAZ1-AVI4	*bla*$_{TEM}$ copy number up from 80 to 160	EnvZ Gln115Arg			>256	16	4	>256	4	128	>32	0.125	16	32	>256	0.06	0.25	1	0.015
***E. coli* J53 TEM-10**	**TEM-10**	**OmpC, OmpF both active**			**>256**	**1**	**0.5**	**256**	**0.5**	**2**	**32**	**0.06**	**1**	**1**	**4**	**0.03**	**0.03**	**0.5**	**0.015**
Mutant 2 CAZ4-AVI1	Mutation upstream of *bla*$_{TEM}$ (-548) regulatory region possibly Affecting expression?				>256	16	4	>256	4	256	>32	0.125	32	>64	>256	0.06	0.25	1	0.015
Mutant 3 CAZ4-AVI1	Mutation upstream of *bla*$_{TEM}$ (-548) regulatory region possibly Affecting expression?				>256	16	4	>256	4	>256	>32	0.25	32	>64	>256	0.06	0.25	0.5	0.015

Mutant 4 CAZ4-AVI1	Mutation upstream of *bla*$_{TEM}$ (-548) regulatory region possibly Affecting expression?				>256	16	4	>256	4	>256	>32	0.125	32	>64	>256	0.06	0.25	0.5	0.015
Mutant 5 CAZ4-AVI1	Mutation upstream of *bla*$_{TEM}$ (-548) regulatory region possibly Affecting expression?	EnvZ : Leu35Gln			>256	32	16	>256	16	>256	>32	0.5	32	>64	>256	0.25	2	1	0.03

Abbreviations: Avi, avibactam; Clav, clavulanate; CP, ciprofloxacin; CTX, cefotaxime, Ert, ertapenem, GENT, gentamicin; MEM, meropenem; PTZ, piperacillin/tazobactam and Taz, tazobactam

[a] Numbering is from the first amino acid of the coding sequence, irrespective of whether this is cleaved as a signal peptide

[b] Selective conditions, CAZ 1 AVI1 means ceftazidime 1 mg/L plus avibactam 1 mg/L

Parent strains are shown in bold font