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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

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Objectives: The objective of this study was to explore the activity of ceftazidime and ceftazidime/avibactam against a collection of isogenic strains of *Escherichia coli* DH10B possessing SHV and KPC β-lactamases containing single amino acid substitutions in the Ω-loop (residues 164–179).

Methods: Ceftazidime and ceftazidime/avibactam MICs were determined by the agar dilution method for a panel of isogenic *E. coli* strains expressing SHV-1 and KPC-2 with amino acid substitutions at positions 164, 167, 169 or 179. Two KPC-2 β-lactamase variants that possessed elevated MICs of ceftazidime/avibactam were selected for further biochemical analyses.

Results: Avibactam restored susceptibility to ceftazidime for all Ω-loop variants of SHV-1 with MICs <8 mg/L. In contrast, several of the Arg164 and Asp179 variants of KPC-2 demonstrated MICs of ceftazidime/avibactam >8 mg/L. β-Lactamase kinetics showed that the Asp179Asn variant of KPC-2 demonstrated enhanced kinetic properties against ceftazidime. The $K_{i\ app}$, k_2/K and k_{off} of the Arg164Ala and Asp179Asn variant KPC-2 β-lactamases indicated that avibactam effectively inhibited these enzymes.

Conclusions: Several KPC-2 variants demonstrating ceftazidime resistance as a result of single amino acid substitutions in the Ω-loop were not susceptible to ceftazidime/avibactam (MICs >8 mg/L). We hypothesize that this observation is due to the stabilizing interactions (e.g. hydrogen bonds) of ceftazidime within the active site of variant β-lactamases that prevent avibactam from binding to and inhibiting the β-lactamase. As ceftazidime/avibactam is introduced into the clinic, monitoring for new KPC-2 variants that may exhibit increased ceftazidime kinetics as well as resistance to this novel antibiotic combination will be important.

Keywords: β-lactamase inhibitors, extended-spectrum β-lactamases, ESBLs, antibiotic resistance

Introduction

β-Lactam antibiotics are highly effective inhibitors of the PBPs in bacteria. The binding of β-lactams to PBPs prevents peptidoglycan cross-linking and eventually leads to cell lysis and death. Bacteria continually evolve in response to antibiotic threats and many Gram-negative bacteria produce β-lactamase enzymes that are able to cleave the β-lactam bond of the antibiotics and render them ineffective.

β-Lactamases are grouped into four classes according to the Ambler system; class A, C and D β-lactamases utilize an active-site serine residue, while class B metallo-β-lactamases possess Zn^{2+} ions as a nucleophile.[1] β-Lactamases are also phenotypically or functionally classified into three groups according to the Bush–Jacoby classification system. The group 1 β-lactamases (class C) hydrolyse cephalosporins more than penicillins as their defining characteristic. The group 2 enzymes (class A and D) hydrolyse penicillins and possess variants with extended-spectrum properties that also hydrolyse oxyimino-cephalosporins. In addition, other variants are present in group 2 that exhibit resistance to β-lactamase inhibitors. The group 3 β-lactamases (class B) confer broad-spectrum hydrolysis with the exception of monobactams.[2,3]

Several conserved motifs are present in class A β-lactamases including the Ω-loop, which encompasses amino acid residues Arg164 to Asp179.[4–10] The Ω-loop is particularly important as it contains two amino acids involved in the acylation and deacylation of substrates by β-lactamases, Glu166 and Asn170

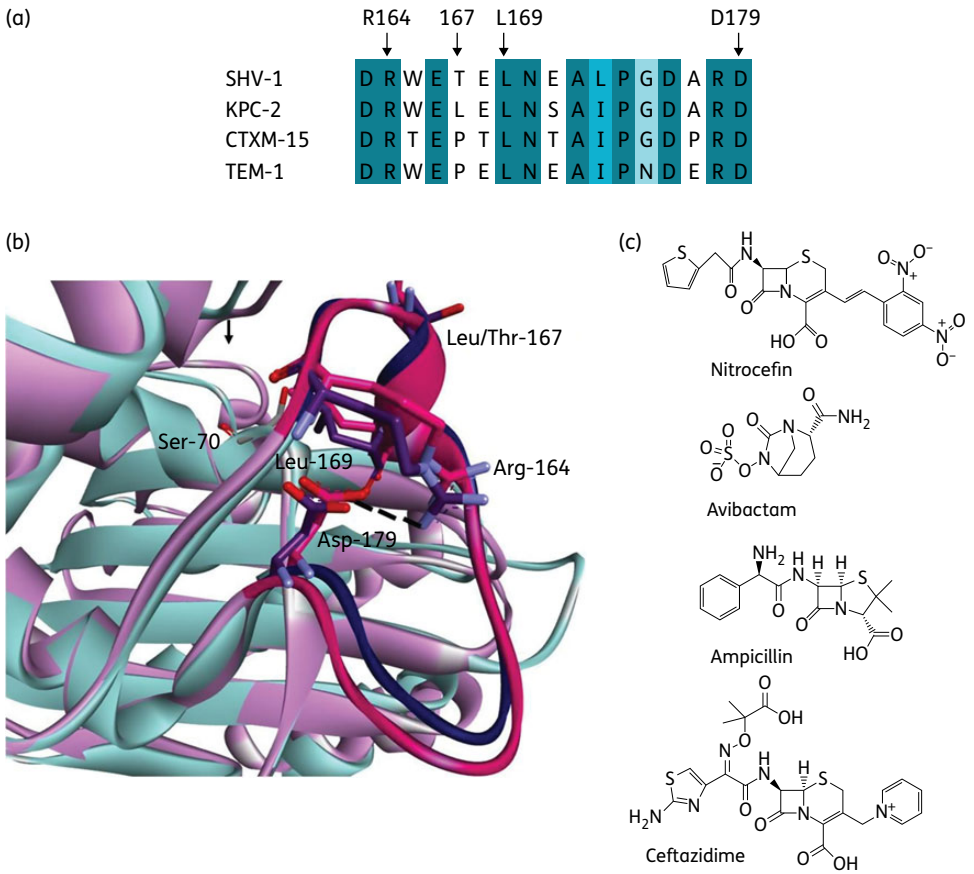

Figure 1. (a) Amino acid sequence alignment of the Ω-loop in four class A β-lactamases. The black arrows mark the sites of focus in this paper (Arg164, Leu/Pro167, Leu169 and Asp179). (b) Overlay of the X-ray crystallographic protein structure of SHV-1 (PDB ID: 1SHV, pink/magenta) and KPC-2 (PDB ID: 2OV5, cyan/purple) showing the Ω-loop in deeper colours with the catalytic Ser70, Arg164, Leu/Thr167, Leu169 and Asp179 labelled and the Arg164–Asp179 salt bridge drawn as a broken black line. (c) Chemical structures of the compounds tested in this paper. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

(Figure 1a and b).[5,6,10–12] Single amino acid substitutions, particularly at positions 164, 167, 169 and 179 within the Ω-loop of class A β-lactamases, lead to reduced susceptibility to ceftazidime (Figure 1b).[5–9,12–19] Enhanced kinetics towards ceftazidime is responsible for the extension of the substrate profile of some TEM-1 and SHV-1 variants at these positions.[12,14,20–28]

Avibactam is a novel non-β-lactam β-lactamase inhibitor that is being developed with ceftazidime for the treatment of Gram-negative bacterial infections. Ceftazidime/avibactam demonstrates potent activity against Enterobacteriaceae possessing extended-spectrum β-lactamases (ESBLs).[29–33] Single amino acid substitutions in the Ω-loop that confer the ESBL phenotype are commonly encountered in clinical isolates (Table 1) and the inhibition of these Ω-loop variants by avibactam has not previously been explored. Thus, we tested to see if avibactam could restore the activity of ceftazidime against several SHV-1 and KPC-2 class A β-lactamase variants possessing single amino acid substitutions in the Ω-loop. Little is known about the interplay of cephalosporins in the active site of ESBLs when coupled with a novel β-lactamase inhibitor from the diazabicyclooctanone class like avibactam. However, other traditional β-lactamase inhibitors (i.e. clavulanic acid, tazobactam, sulbactam) have shown activity in partnership with a penicillin or cephalosporin in clinical and non-clinical settings against Ω-loop ESBL SHV variants.[18,34,35]

Table 1. Clinical Ω-loop variants of the SHV and KPC β-lactamases[a]

Enzyme	Amino acid substitution
SHV-6	D179A
SHV-8	D179N
SHV-16	5 amino acid duplication at E166
SHV-19, -20, -21	L173F
SHV-24	D179G
SHV-51	G175A
SHV-57	L169R
SHV-79	A172V
SHV-111	P174S
SHV-143	R164L
KPC-12	L169M

[a]Obtained from the Lahey website: http://www.lahey.org/Studies.

We discovered that avibactam is able to inhibit the Ω-loop variant β-lactamases, as evidenced by comparable inhibitory kinetic parameters for selected KPC variants. Yet ceftazidime/avibactam

MICs were still higher for certain KPC variants with Ω-loop amino acid substitutions. We advance that resistance to ceftazidime/avibactam may be due to enhanced kinetics against ceftazidime (e.g. lower K_m), as previously described for the R164S variant of KPC-2, thus preventing avibactam from inhibiting the enzyme.[15]

Materials and methods

Site-directed mutagenesis

The Agilent site-directed mutagenesis XL kit™ was used to create codon changes corresponding to particular amino acid substitutions at positions 164, 167, 169 and 179 in the bla_{SHV-1} gene in the pBC SK(−) and the bla_{KPC-2} gene in the pBR322-*catI* plasmid. The cloning of these β-lactamase genes into their respective plasmids was previously described.[36,37] McLab (http://www.mclab.com/) was used to sequence each plasmid-encoded β-lactamase gene to verify the success of the mutagenesis reaction.

MIC measurement

Agar-dilution MICs were determined according to the CLSI protocol.[38] Briefly, Mueller–Hinton (M–H) agar was used to pour plates with doubling dilutions of antibiotics. Bacteria were grown overnight in M–H broth and then diluted and stamped onto the plates with a Steers™ replicator to deliver 10 μL of a 10^4 bacterial load per spot. The following day, the plates were read and the MIC was defined as the antibiotic concentration at which bacterial growth was no longer observed. We also performed a second set of MICs using *Escherichia coli* clones containing bla_{SHV-1} and bla_{KPC-2} on LB agar in order to compare values obtained on these two different agar compositions. In addition to agar-dilution MICs, ceftazidime Etest (bioMérieux Diagnostics) MIC assays were determined using the manufacturer's instructions. Broth microdilution using frozen panels (ThermoFisher Scientific, Cleveland, OH, USA) with ceftazidime according to CLSI guidelines and MicroScan panels were also conducted.

Compounds

Ceftazidime was purchased from Sigma-Aldrich, Research Products International Corp. and its commercial source. Avibactam was acquired through a research contract with AstraZeneca Pharmaceuticals. Nitrocefin was purchased from Becton-Dickinson.

β-Lactamase preparation

Four litres of super-optimal broth supplemented with chloramphenicol at 20 mg/L for plasmid maintenance were grown overnight at 37°C with *E. coli* DH10B cells containing pBR322-*catI*-bla_{KPC-2}, pBR322-*catI*-$bla_{KPCArg164Ala}$ or pBR322-*catI*-$bla_{KPCAsp179Asn}$. The cells were pelleted and the supernatant was discarded. The cell pellets were frozen at −20°C. The pellets were thawed, resuspended in Tris–HCl, pH 7.4 with lysozyme, benzonuclease, $MgSO_4$ and EDTA added as previously described.[39] The cellular debris was then pelleted and the supernatant was filtered twice through a Corning 28 mm 0.20 μm syringe filter and these crude periplasmic extracts were used for enzyme kinetic analysis. We note that, during attempted procedures to obtain pure enzyme, these Ω-loop variants were unstable. In addition, the variants were not expressed equally at steady-state; therefore, the amount of extract used for kinetic analysis was normalized to the level of nitrocefin hydrolysis.[15]

Steady-state enzyme kinetic measurements

All experiments were performed at room temperature (25°C) in 10 mM PBS pH 7.4 using crude extracts under steady-state conditions on an Agilent 8453 spectrophotometer as previously described with all values having an error of 10%.[40] Due to the use of the periplasmic extracts, all values are reported as the apparent kinetic constants. For nitrocefin, the apparent K_m and V_{max} were determined by measuring initial velocities at a variety of nitrocefin concentrations. Then, the data were fitted to the Michaelis–Menten equation (non-linear least-square fit) using EnzFitter to obtain the apparent V_{max} and K_m.

For avibactam, K_i apparent or $K_{i\ app}$ was determined by a competition assay with nitrocefin as previously reported.[41] Briefly, increasing concentrations of avibactam (from 2.5 to 20 μM) were added to extract/nitrocefin mixtures and the initial velocities (v_i) were measured. Excel was used to plot $1/v_i$ versus the concentration of avibactam and the data were fitted to a linear equation, where the *y*-intercept divided by the slope of the line was defined as the $K_{i\ app\ observed}$. This value was corrected for the use of nitrocefin according to equation 1 and this corrected value was defined as $K_{i\ app}$.

$$K_{i\ app} = \frac{K_{i\ app\ observed}}{1 + [\text{nitrocefin}]/K_{m\ nitrocefin}} \quad (1)$$

The apparent onset of acylation (k_2/K) was approximated for avibactam as previously described.[41,42] Briefly, the apparent k_2/K values were determined by measuring timed inactivation of the periplasmic extracts using 100 μM of nitrocefin as a reporter substrate and increasing concentrations of avibactam over a 400 s time course. Origin 8.1 was used to fit each time course to equation 2 to obtain a k_{obs} value for each avibactam concentration. Here, v_f is final velocity and A_i is the initial absorbance at $\lambda = 482$ nm.

$$y = v_f * x + (v_i - v_f) * \frac{1 - e^{-k_{obs} * x}}{k_{obs}} + A_i \quad (2)$$

Then, the k_{obs} values were plotted against [avibactam] and fitted to a linear equation using Excel. The k_2/K observed was derived using equation 3. The k_2/K observed was then corrected for the use of nitrocefin to obtain the apparent k_2/K value according to equation 4.

$$k_{obs} = k_{-2} + \frac{k_2}{K} * \frac{[\text{avibactam}]}{1 + [\text{nitrocefin}]/K_m} \quad (3)$$

$$\frac{k_2}{K} = \frac{k_2}{K}(\text{observed}) * [\text{nitrocefin}]/K_{m\ nitrocefin} + 1 \quad (4)$$

The apparent k_{off} was determined as previously described.[41,42] Periplasmic extracts were incubated with an excess of avibactam (as determined by full enzyme inhibition using nitrocefin as a reporter substrate) for 5 min. Serial dilutions of the enzyme/inhibitor mixes were performed to eventually dilute the mixture 1:1000 with 100 μM nitrocefin as a reporter substrate and the hydrolysis was measured for 3600 s. The apparent k_{off} was calculated by fitting the resulting curve to a single exponential equation using Excel.

Ceftazidime (50 μM) hydrolysis curves were measured at $\lambda = 260$ nm using periplasmic extracts of KPC-2 and the KPC-2 Asp179Asn variant at room temperature (25°C) in PBS during a 100 s time course. The lines were compared with background spontaneous hydrolysis of ceftazidime alone and fitted to a simple linear equation in Excel to obtain the rate of hydrolysis as the slope of the line.

Rapid-mixing stopped-flow kinetics

Pre-steady-state kinetics were measured on an Applied Photophysics SX-20 Stopped-Flow spectrophotometer using similar conditions as for the ceftazidime hydrolysis curves described above. However, for stopped-flow kinetics, a cuvette with a 0.2 cm pathlength was used and data were collected on a logarithmic scale measuring 1000 points over 1 s.

Results

Susceptibility to ceftazidime/avibactam combinations

The MIC measurements for the Ω-loop variants of SHV-1 and KPC-2 expressed in *E. coli* DH10B are shown in Table 2. Many of the SHV-1

Table 2. MICs in mg/L for various Ω-loop mutants of KPC and SHV tested with ceftazidime and ceftazidime/avibactam on M–H agar[a,b]

Strain	Ceftazidime	Ceftazidime/avibactam
E. coli DH10B	0.25	0.25
E. coli DH10B pBC SK(−) empty[c]	0.5	0.25
E. coli DH10B pBC SK(−) bla_{SHV-1}	4–8	0.5
E. coli DH10B pBC SK(−) $bla_{SHV\ Arg164Ala}$	128	0.5
E. coli DH10B pBC SK(−) $bla_{SHV\ Arg164His}$	64	0.5
E. coli DH10B pBC SK(−) $bla_{SHV\ Arg164Pro}$	32	0.5
E. coli DH10B pBC SK(−) $bla_{SHV\ Thr167Ala}$	8	0.5
E. coli DH10B pBC SK(−) $bla_{SHV\ Thr167Leu}$	8	0.5
E. coli DH10B pBC SK(−) $bla_{SHV\ Thr167Pro}$	0.5	0.5
E. coli DH10B pBC SK(−) $bla_{SHV\ Leu169Ala}$	4	0.25
E. coli DH10B pBC SK(−) $bla_{SHV\ Leu169Arg}$	64	0.5
E. coli DH10B pBC SK(−) $bla_{SHV\ Asp179Ala}$	64	0.5
E. coli DH10B pBC SK(−) $bla_{SHV\ Asp179Gln}$	32	0.5
E. coli DH10B pBC SK(−) $bla_{SHV\ Asp179Asn}$	32	0.5
E. coli DH10B pBR322-*catI*-bla_{KPC-2}	64	1
E. coli DH10B pBR322-*catI*-$bla_{KPC\ Arg164Ala}$	256	16
E. coli DH10B pBR322-*catI*-$bla_{KPC\ Arg164His}$	256	8
E. coli DH10B pBR322-*catI*-$bla_{KPC\ Arg164Pro}$	256	64
E. coli DH10B pBR322-*catI*- $bla_{KPC\ Arg164Ser}$	256	8
E. coli DH10B pBR322-*catI*-$bla_{KPC\ Leu167Ala}$	32	1
E. coli DH10B pBR322-*catI*-$bla_{KPC\ Leu167Pro}$	16	1
E. coli DH10B pBR322-*catI*-$bla_{KPC\ Leu167Thr}$	32	1
E. coli DH10B pBR322-*catI*-$bla_{KPC\ Leu169Ala}$	128	2
E. coli DH10B pBR322-*catI*-$bla_{KPC\ Leu169Arg}$	256	0.5
E. coli DH10B pBR322-*catI*-$bla_{KPC\ Asp179Ala}$	512	64
E. coli DH10B pBR322-*catI*-$bla_{KPC\ Asp179Gln}$	512	32
E. coli DH10B pBR322-*catI*-$bla_{KPC\ Asp179Asn}$	>512	32

[a]Avibactam added to each plate at 4 mg/L.
[b]MICs determined on LB agar for WT bla_{SHV-1} and bla_{KPC-2} encoded in the same respective vectors were 2 and 32 mg/L.[15,43]
[c]The empty vector refers to the pBC SK(−) phagemid without a *bla* gene insertion.

Ω-loop variants raised the ceftazidime MIC from 4–8 mg/L for WT SHV-1 to 32–128 mg/L. Similarly, several of the KPC-2 Ω-loop variants increased the ceftazidime MIC from 64 mg/L for WT KPC-2 to 256 and >512 mg/L. When avibactam was added to ceftazidime, the MICs were lowered for all of the bacterial strains. Avibactam decreased the ceftazidime MICs to 0.5 mg/L or lower for all of the SHV-1 variants. However, ceftazidime/avibactam MICs remained >8 mg/L for five of the KPC-2 variants—Arg164Ala (16 mg/L), Arg164Pro (64 mg/L), Asp179Ala (64 mg/L), Asp179Gln (32 mg/L) and Asp179Asn (64 mg/L)—while the ceftazidime/avibactam MIC for WT KPC-2 was lowered to 1 mg/L.

Potency of ceftazidime preparations and MIC creep

The ceftazidime MIC measurements that we obtained for *E. coli* DH10B expressing pBC SK(−) with bla_{SHV-1} (4–8 mg/L) and pBR322-*catI*- with bla_{KPC-2} (64 mg/L) are higher than those previously reported by our laboratory: 1–2 mg/L for SHV-1 and 32 mg/L for KPC-2.[15,43] To address these higher ceftazidime MICs, we repeated ceftazidime MICs using three different frozen stocks of *E. coli* DH10B pBC SK(−) bla_{SHV-1} with three different formulations of ceftazidime. We obtained MICs between 4 and 8 mg/L for all measurements. Ceftazidime Etest MICs were also conducted and an MIC of 4 mg/L was observed (see the Acknowledgements section). Broth microdilution MICs using frozen panels from Thermofisher Scientific and MicroScan also produced results of 4–8 mg/L for ceftazidime.

However, the previously reported MICs used LB agar for determination of the agar dilution MICs whereas we used M–H agar for our studies. Therefore, we performed a comparison of LB and M–H agar for MIC determination. When agar dilution MICs were performed with the LB agar, we determined MICs of 2–4 mg/L for the SHV-1 clone and 32 mg/L for the KPC-2 clone. Therefore, we believe that our higher MICs are due to the characteristics of the M–H agar. The MIC comparison of the different variant enzymes is valid as all MICs were performed using M–H agar.

Biochemical assays for avibactam inhibition and ceftazidime hydrolysis by KPC-2 and selected variants

To begin to understand the mechanistic details behind the elevated ceftazidime/avibactam MICs in the five KPC Ω-loop variants, we selected the Arg164Ala and Asp179Asn variants for further biochemical assays with avibactam and ceftazidime. We used

Table 3. Enzyme kinetics of periplasmic extracts of KPC variants

	KPC-2	KPC-2 Arg164Ala	KPC-2 Asp179Asn
Nitrocefin			
K_m (μM)	15 ± 4	118 ± 50	5 ± 1
V_{max}/K_m (s^{-1})	0.06	0.005	0.09
Avibactam			
$K_{i\ app}$ (μM)	1.2 ± 0.1	2.2 ± 0.2	0.4 ± 0.04
k_2/K ($M^{-1}\ s^{-1}$)	17000	7000	38000
k_{off}	1.1×10^{-4}	4.9×10^{-5}	4.9×10^{-5}

crude β-lactamase extracts to perform inhibitory enzyme kinetics on these β-lactamase variants as the purified proteins were unstable under our purification conditions.

Nitrocefin kinetic assays were performed initially to assess the catalytic activity of these variants. The apparent K_m for nitrocefin for the Asp179Asn variant was similar to WT KPC-2, but the Arg164Ala variant had a 10-fold higher K_m for nitrocefin (Table 3). Additionally, the V_{max}/K_m ratios were similar for the Asp179Asn variant and KPC-2, but 10-fold lower for the Arg164Ala variant.

To assess the inhibitory capacity of avibactam against the Arg164Ala and Asp179Asn variants, the $K_{i\ app}$ and k_2/K values were determined. Similar concentrations of avibactam were necessary to obtain full inhibition of each enzyme variant (Figure 2). The variants were rapidly acylated with a k_2/K value lowest for the Arg164Ala variant and highest for the Asp179Asn variant (Table 3). The $K_{i\ app}$ was similar among the three enzymes (Table 3). The k_{off} was also slow for all three variant enzymes (Table 3).

To evaluate the impact of the single amino acid substitutions in the Ω-loop on ceftazidime hydrolysis, extracts of KPC-2 and the Asp179Asn variant were incubated with ceftazidime during two different time courses (Figure 3). During a 100 s time course, the ceftazidime hydrolysis rates were similar between the two enzymes. However, using rapid-mixing stopped-flow spectroscopy during a 1.5 ms time course, a 'burst' in ceftazidime hydrolysis was observed with the Asp179Asn variant, but not with KPC-2. The level of ceftazidime hydrolysis by KPC-2 was similar to the background level of spontaneous ceftazidime hydrolysis under these pre-steady-state conditions.

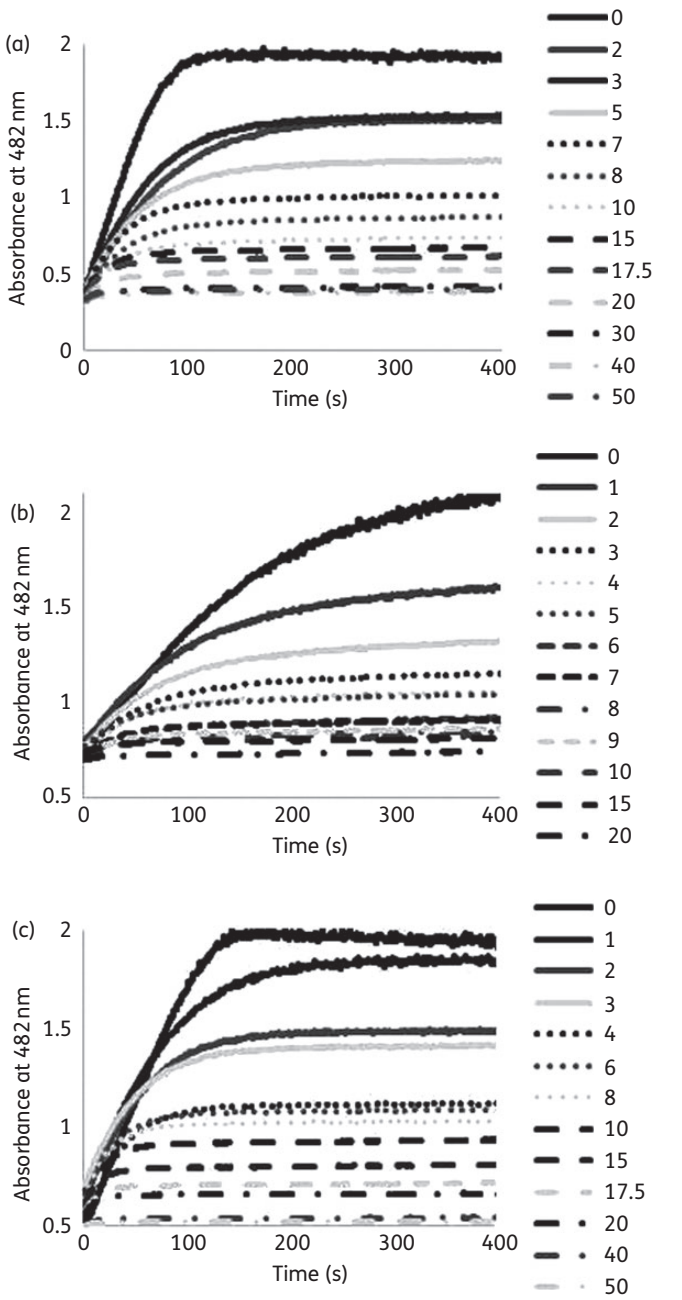

Figure 2. (a) Inhibition of nitrocefin (100 μM) hydrolysis by KPC-2 with increasing concentrations of avibactam (2–50 μM). (b) Inhibition of nitrocefin (100 μM) hydrolysis by KPC Arg164Ala with increasing concentrations of avibactam (1–20 μM). (c) Inhibition of nitrocefin (100 μM) hydrolysis by KPC Asp179Asn with increasing concentrations of avibactam (1–50 μM).

Discussion

Variant class A β-lactamases with single amino acid substitutions in the Ω-loop pose a significant threat against expanded-spectrum cephalosporins as these enzymes hydrolyse ceftazidime and other oxyimino-cephalosporins at a greater rate than their parent enzyme. We showed that single amino acid substitutions at positions 164, 169 and 179 of SHV-1 and KPC-2 raised the ceftazidime MIC for isogenic strains of *E. coli* containing these variant enzymes. Notably, we did not observe elevated ceftazidime MICs for strains carrying 167 variants. Conversely, substitutions at position 167 in the CTX-M or TEM class A β-lactamases were shown to convey increased ceftazidime MICs.[6,17,19] The addition of avibactam to ceftazidime was able to reduce ceftazidime MICs for all of the variants with single amino acid substitutions in the Ω-loops of SHV-1 and KPC-2. However, five of the KPC-2 variants demonstrated ceftazidime/avibactam MICs >8 mg/L.

Notably, ceftazidime MICs were higher for *E. coli* DH10B pBC SK(−) expressing SHV-1 and pBR322-*catI*- producing KPC-2 than previously reported by our group.[15,43] After testing multiple formulations of ceftazidime using three different frozen stocks of *E. coli* DH10B pBC SK(−) expressing SHV-1, we concluded that

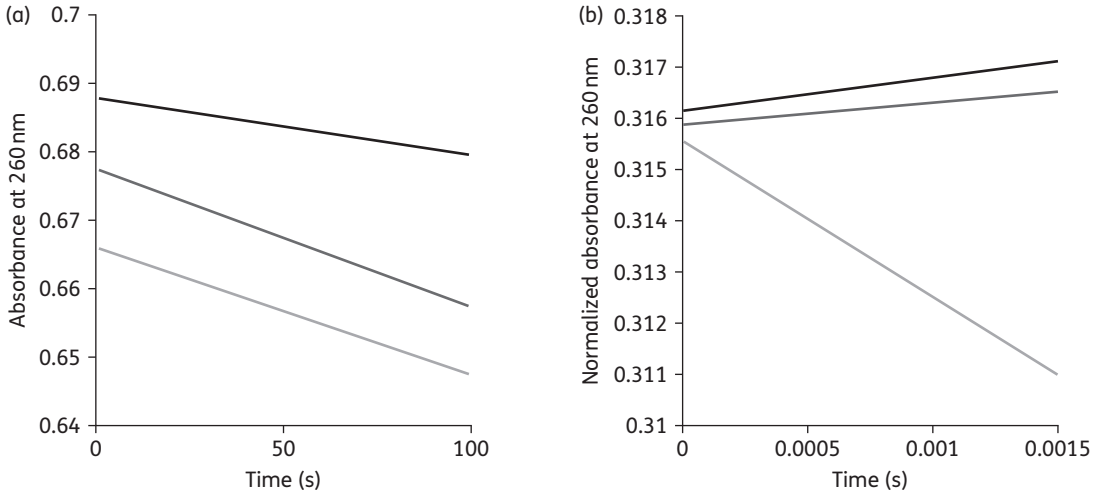

Figure 3. (a) Hydrolysis of ceftazidime (50 μM) by periplasmic extracts of KPC-2 (dark grey) and Asp179Asn (light grey); ceftazidime alone (black) during a 100 s time course. (b) Hydrolysis of ceftazidime (50 μM) by periplasmic extracts of KPC-2 (dark grey) and Asp179Asn (light grey); ceftazidime alone (black) during a 1.5 ms time course.

the difference can be attributed to the use of M–H agar in this study versus LB agar in previous work.[15,43] As three different frozen stocks of bacteria were used for this analysis, we did not believe that this was an issue with our clones. However, we also verified each clone by DNA sequencing and found the *bla* genes and promoters to be identical. Further studies will be completed to evaluate the effect of the different agar formulations on MIC determination.

Selected variants of KPC-2 with elevated MICs to ceftazidime/avibactam were assayed kinetically with avibactam and ceftazidime. The inhibitory avibactam kinetics were similar between KPC-2 and the Arg164Ala and Asp179Asn variants, indicating that avibactam was able to encounter and inactivate these β-lactamases with similar potency. The ceftazidime kinetics revealed that the KPC-2 and Asp179Asn variant hydrolysed ceftazidime at a similar rate during a 100 s study period. Conversely, rapid-mixing stopped-flow kinetics of KPC-2 and the Asp179Asn variant on a 1.5 ms timescale showed a phenomenon that was previously observed with the Arg164Ser variant of KPC-2 and ceftazidime.[15] The Arg164Ser variant showed a 'burst', where ceftazidime was rapidly bound to the variant enzyme and unable to interact with its PBP target. This 'burst' leads to ceftazidime resistance despite comparable hydrolysis rates among the enzymes in steady-state experiments due to an increased affinity of ceftazidime for the enzyme. We qualitatively observed a 'burst' with the Asp179Asn variant. Thus, we postulate that increased affinity for ceftazidime may prevent binding of avibactam to our enzymes with Ω-loop substitutions, resulting in elevated ceftazidime/avibactam MICs despite potent avibactam inhibition of these enzymes. Since avibactam has not been shown to have activity on its own, the PBP targets are not inactivated. As a result, elevated ceftazidime/avibactam MICs were observed. Further biochemical analyses of Ω-loop variants of KPC-2 are currently underway to dissect their mechanisms of ceftazidime resistance and will be the focus of separate studies.

One KPC-2 variant with an amino acid substitution in the Ω-loop has emerged clinically (KPC-12; Table 1). Thus, the appearance of bacteria containing Ω-loop variants of KPC-2 may threaten the efficacy of ceftazidime/avibactam combination therapy. However, since there appears to be a severe evolutionary cost of Ω-loop amino acid substitutions that results in impaired stability, we hypothesize that our tested variants may not emerge clinically without a secondary stabilizing substitution (i.e. a 'global suppressor' substitution).[4–7,44,45] Evidence of global suppressor mutations in the class A TEM-1 β-lactamase has been well established; their role in KPC-2 is not yet defined.[44,45] As ceftazidime/avibactam is introduced into the clinic, being aware of their limitations of efficacy against ESBL enzyme variants with high ceftazidime resistance is essential.

Conclusions

Here, we revealed that resistance to ceftazidime/avibactam might occur via mutations to KPC enzymes that result in enhanced ceftazidime kinetics rather than reduced avibactam inhibition. The likelihood of this as a clinical risk remains to be ascertained. This is in contrast to typical inhibitor resistant class A β-lactamases, which commonly manifest this phenotype as a result of resistance to the inhibitor mediated by changes in the amino acid sequence.[46,47] All class A β-lactamase variants resistant to the commercially available combinations of amoxicillin/clavulanic acid, ampicillin/sulbactam and piperacillin/tazobactam are resistant due to inhibitor-resistant substitutions (e.g. substitutions at positions 69, 130 and 234), not due to increased resistance to the β-lactam partner.[46–48] Here, Ω-loop variants are more resistant to ceftazidime/avibactam, due to ESBL substitutions (e.g. Ω-loop) that alter ceftazidime kinetics. This lowers the effectiveness of the ceftazidime/avibactam combination but does not lead to inhibitor resistance due to an effect on avibactam kinetics. Clinicians will be administering ceftazidime/avibactam to patients who possess infections with ceftazidime-resistant strains; if these ceftazidime-resistant strains also contain certain Ω-loop substitutions, the efficacy of the combination may be in question. Being aware of the emergence of variants with enhanced ceftazidime kinetic properties as the ceftazidime/avibactam combination completes Phase III clinical trials and begins to be introduced

for the treatment of complicated clinical infections is important. These data also stress the importance of ceftazidime in this novel β-lactam/β-lactamase inhibitor combination.

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