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Selection and molecular characterization of ceftazidime/avibactam-resistant mutants in *Pseudomonas aeruginosa* strains containing derepressed AmpC

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Objectives: *Pseudomonas aeruginosa* is an important nosocomial pathogen that can cause a wide range of infections resulting in significant morbidity and mortality. Avibactam, a novel non-β-lactam β-lactamase inhibitor, is being developed in combination with ceftazidime and has the potential to be a valuable addition to the treatment options for the infectious diseases practitioner. We compared the frequency of resistance development to ceftazidime/avibactam in three *P. aeruginosa* strains that carried derepressed *ampC* alleles.

Methods: The strains were incubated in the presence of increasing concentrations of ceftazidime with a fixed concentration (4 mg/L) of avibactam to calculate the frequency of spontaneous resistance. The mutants were characterized by WGS to identify the underlying mechanism of resistance. A representative mutant protein was characterized biochemically.

Results: The resistance frequency was very low in all strains. The resistant variants isolated exhibited ceftazidime/avibactam MIC values that ranged from 64 to 256 mg/L. All of the mutants exhibited changes in the chromosomal *ampC* gene, the majority of which were deletions of various sizes in the Ω-loop region of AmpC. The mutant enzyme that carried the smallest Ω-loop deletion, which formed a part of the avibactam-binding pocket, was characterized biochemically and found to be less effectively inhibited by avibactam as well as exhibiting increased hydrolysis of ceftazidime.

Conclusions: The development of high-level resistance to ceftazidime/avibactam appears to occur at low frequency, but structural modifications in AmpC can occur that impact the ability of avibactam to inhibit the enzyme and thereby protect ceftazidime from hydrolysis.

Keywords: extended-spectrum cephalosporinases, ESAC, Ω-loop, cephalosporins

Introduction

The clinical management of serious hospital infections caused by Gram-negative bacterial pathogens is becoming increasingly difficult owing to the constant erosion of treatment options. The level of bacterial resistance has increased significantly, which has rendered many classes of antibacterial drugs obsolete.[1–3] *Pseudomonas aeruginosa* is an opportunistic pathogen and hospital-acquired infections caused by this organism are a significant medical problem, especially in patients with compromised immune systems, and it represents one of the most common species associated with healthcare-associated infections.[4] Empirical treatment of *P. aeruginosa* infections typically comprises a combination of antibacterial agents, although the increasing prevalence of β-lactamase enzymes has significantly decreased the effectiveness of β-lactam drugs, once the foundation of an antipseudomonal treatment regimen. β-Lactamase enzymes can be encoded on transmissible plasmids, but *P. aeruginosa* represents one species wherein the chromosomally encoded class C enzyme can be induced in the presence of β-lactams and can also become stably derepressed leading to β-lactam resistance.[5–8] Overexpression of the AmpC cephalosporinase can result in resistance to currently used treatment agents such as ceftazidime and cefepime.[5] Clinically used β-lactamase inhibitors, used in combination with β-lactam drugs, have an inhibition profile limited to class A enzymes and are unable to inhibit the

AmpC β-lactamases, therefore offering no benefit to the primary β-lactam treatment as the hydrolytic capacity of AmpC remains intact.

Avibactam is a novel non-β-lactam β-lactamase inhibitor with activity against both class A and class C as well as some class D enzymes and is currently in clinical development with ceftazidime, ceftaroline fosamil and aztreonam. The combination of ceftazidime and avibactam has been shown to be effective against most isolates of *P. aeruginosa*, including those that overexpress the chromosomal AmpC β-lactamase.[9] Structural and kinetic studies have provided critical insights about the broad-spectrum activity and reversible inhibitory property of this compound.[10–13] The *P. aeruginosa* AmpC enzyme exhibits natural sequence variations that can influence the hydrolytic efficiency against ceftazidime,[8] although a conservational analysis of >500 geographically diverse clinical isolates from a wide range of clinical indications has demonstrated that the key residues responsible for binding avibactam are very highly conserved.[13] There are reports of Enterobacteriaceae spp. that have developed resistance to ceftaroline/avibactam[14] or aztreonam/avibactam,[13] yet to date there have been no published studies on the propensity of *P. aeruginosa* to develop resistance to ceftazidime/avibactam. In this study, *P. aeruginosa* strains containing the *ampC* gene derepressed to various levels were challenged with ceftazidime/avibactam to determine the frequency of spontaneous resistance as well as to identify any mutation that would affect the ability of avibactam to adequately protect ceftazidime from hydrolysis. Additionally, *P. aeruginosa* resistant variants isolated from an *in vitro* pharmacokinetic (PK)/pharmacodynamic (PD) hollow-fibre system were also characterized, which together allowed an understanding of the alterations that result in weaker inhibitory activity of this drug combination.

Materials and methods

Bacterial strains

The ceftazidime-resistant *P. aeruginosa* isolates ARC3608 and ARC3610 used in this study were obtained from a ceftazidime/avibactam surveillance study and were provided by JMI Laboratories (North Liberty, IA, USA). *P. aeruginosa* ARC3509 (ceftazidime resistant) was obtained from Novexel (France) and the control isolate ATCC 27853 was obtained from ATCC (Manassas, VA, USA).

Antimicrobial susceptibility testing

The MIC of each compound for each isolate was determined using either the broth microdilution method or the agar dilution method following CLSI guidelines. All compounds were tested in accordance with CLSI recommendations. *P. aeruginosa* ATCC 27853 was used as the quality control isolate. All reference compounds were obtained from US Pharmacopeial Convention (Rockville, MD, USA) with the exception of doripenem (Sigma, St Louis, MO, USA) and aztreonam (MP Biomedicals, Santa Ana, CA, USA). Ceftazidime and avibactam were obtained from AstraZeneca (manufactured by GlaxoSmithKline). To determine the ceftazidime/avibactam MIC values, the avibactam concentration was fixed at 4 mg/L while the ceftazidime concentration was varied.

In vitro PK/PD

The conditions and design of the *in vitro* PK/PD hollow-fibre experiment have been described elsewhere.[15] The strain ARC3610, referred to as 1388,[15] was one isolate that demonstrated regrowth after 24 h when exposed to simulated human exposures of ceftazidime/avibactam.

Determination of spontaneous resistance frequency

Microorganisms were harvested from blood agar plates (containing sheep red blood cells; Remel, Lenexa, KS, USA) that had been grown overnight and suspended in Mueller–Hinton 2 broth (MHB2) (Sigma) to an OD_{600} of ∼3.2, which corresponds to a density of $\sim10^9$–10^{10} cfu/mL. A dilution series of this suspension was plated to determine the number of cfu in the original suspension. In addition, 100 μL volumes of the suspension were spread evenly, in triplicate, onto Mueller–Hinton agar (BD, Franklin Lakes, NJ, USA) plates containing ceftazidime/avibactam. The avibactam concentration was maintained at 4 mg/L and ceftazidime was varied at 2-fold concentrations relative to the agar dilution MIC value. The plates were incubated for 24 h at 36°C. The numbers of colonies growing on plates at multiples of the agar dilution MIC were counted after 24 h and resistance frequencies calculated. Representative variants were then passed once on selective media and twice on drug-free media and tested for changes in MIC using a standard broth microdilution assay following CLSI guidelines. Frequencies of resistance at 2-, 4- and 8-fold the agar dilution MIC were calculated as the ratio of the number of resistant variants observed on the compound-containing agar plates compared with the total number of viable cells plated. The latter was calculated from a serial dilution series of the original suspension performed on compound-free agar plates. The selection plates were held at 36°C for a further 24 h to evaluate if any additional resistant variants emerged.

RNA analysis

RNA was harvested from logarithmically growing bacterial cells. Colonies were taken from a blood agar plate (Remel) and inoculated into 5 mL of MHB2 broth (Sigma) and grown at 37°C with shaking until an OD_{600} of ∼0.8 was achieved. Cells were treated with RNAprotect (Qiagen, Valencia, CA, USA) and harvested by centrifugation. RNA was extracted with a Maxwell 16 RNA Kit (Promega, Madison WI, USA). RT–PCR was performed on a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Berkeley, CA, USA) using the Qiagen QuantiTect SYBR Green RT–PCR Kit (Qiagen). The oligonucleotides used to detect *ampC* expression were 5′-GATACCAGATTCCCCTGC-3′ and 5′-GGTTCTCCTTTCAGGCTG-3′. Those to detect the *rpsL* ribosomal gene used for normalization purposes were 5′-GCTGCAAAACTGCCCGCAACG-3′ and 5′-ACCCGAGGTGTCCAGCGAACC-3′.

WGS

Genomic DNA was purified on the Maxwell 16 platform (Promega) and quantified using a Qubit fluorometer (Invitrogen Life Technologies, Grand Island, NY, USA). DNA libraries were prepared using the Nextera library construction protocol (Illumina, San Diego, CA, USA) following the manufacturer's instructions and sequenced on a MiSeq Sequencer (Illumina). For each isolate, ∼2.5 million 150 bp paired-end sequence reads were assembled and analysed using the CLCBio suite of software tools (Cambridge, MA, USA) to identify regions of difference.

Cloning, expression and purification of AmpC proteins

The expression plasmids pJT1092 and pJT1093 were constructed by amplifying the *ampC* gene by PCR, using primers PaeAmpC-NdeI-For (5′-GGACAGCATATGCGCGATACCAGATTC-3′) and PaeAmpC-BamHI-Rev (5′-ATTGATGGATCCTCAGCGCTTCAGCGGCACCTTGGC-3′), from genomic DNA isolated from 3610-C5 and ARC3610, respectively, with High Fidelity PCR Master (Roche Applied Science) using reaction conditions specified by the manufacturer. Both the purified PCR product and the pET-30a expression vector (EMD Millipore, Billerica, MA, USA) were digested with NdeI and

BamHI, ligated and transformed into *Escherichia coli* DH5α-T1^R chemically competent cells (Life Technologies, Grand Island, NY, USA). Transformants were selected on LB agar plates supplemented with 25 mg/L kanamycin. Plasmids were isolated and verified by PCR and sequencing. *E. coli* BL21(DE3) cells transformed with plasmid pJT1092 or pJT1093 were inoculated into LB medium supplemented with 25 mg/L kanamycin at $OD_{600}=0.1$, incubated at 37°C for 2 h, induced with 0.1 mM IPTG at OD_{600} ~0.7 and incubated for 22 h at room temperature. The cells were harvested by centrifugation and the AmpC proteins purified as described previously.[11,12]

Biochemical characterization of AmpC proteins

Enzyme measurements were made at 37°C in buffer consisting of 50 mM sodium phosphate (pH 7) supplemented with 0.01% Triton X-100. K_m and k_{cat} determinations for penicillin G were made via full progress curve analysis,[8] using the program KinTek Global Kinetic Explorer version 2.4 (KinTek, Austin, TX, USA). Due to the very low rates of ceftazidime hydrolysis, K_m values for ceftazidime were determined as the K_i in competition with nitrocefin.[16,17] Competition studies were made as initial rates collected within the first 2 min of reactions and were carried out at 1× K_m of nitrocefin (0.1 mM) for each of the AmpC proteins. Avibactam IC_{50} values were determined using 20 point doubling dilutions from a top (final) concentration of 200 μM. Compound and enzyme solutions were incubated for 5 min prior to initiation with nitrocefin at 0.1 mM. Final enzyme concentrations were 0.2 nM (AmpC-PAO1), 0.1 nM (AmpC-3610) or 125 nM (AmpC-3610-C5). After initiation, absorbance time course data were collected at 490 nm with sampling every 30 s. The percentage inhibition was calculated from progress curve slopes during the first 3 min using the equation $\%I=\{1-[v/(V_{max}-V_{min})]\}\times100$ where v is the slope of the test well, V_{max} is the slope of the uninhibited control and V_{min} is the slope of the fully inhibited (no enzyme) control. IC_{50} values were calculated from %I data by fitting the data to a four parameter logistic function using XLfit version 5.1 (IDBS, Burlington, MA, USA).

Molecular modelling

The structures of *P. aeruginosa* AmpC, in the native form (PDB:4GZB) and in complex with avibactam (PDB:4OOY), were used to support the structural interpretations. The ceftazidime-bound structure of *E. coli* AmpC (PDB:1IEL) was used to analyse the structure of the substrate-bound conformation. The AmpC mutations identified in this study were mapped onto the known structures using Pymol (Schrödinger, www.pymol.org).

Results

Characterization of P. aeruginosa isolates

The β-lactamase gene content of the three *P. aeruginosa* isolates that were resistant to ceftazidime, ARC3610, ARC3509 and ARC3608, was determined by WGS. These isolates were selected because they showed elevated ceftazidime MIC values (64, 128 and 128 mg/L, respectively) compared with a ceftazidime-susceptible strain with an MIC of 2 mg/L; however, the susceptibility to ceftazidime/avibactam was increased significantly by 8–32-fold in all three isolates. No other clinically important β-lactamase genes were identified in these strains other than the chromosomal bla_{AmpC} allele; bla_{OXA-50} was also observed but this is not important in resistance to clinically used antibacterial β-lactams.[18] This suggested that an overexpressed AmpC enzyme, which could be inhibited by avibactam, was responsible for the ceftazidime resistance. RT–PCR analyses from logarithmically growing cultures were performed to confirm the extent to which the bla_{AmpC} allele was derepressed, which was normalized using the highly expressed *rpsL* ribosomal gene. *P. aeruginosa* ARC3608 had the highest level of bla_{AmpC} expression with an *ampC/rpsL* ratio of 11.6. Isolate ARC3509 had a moderate level of bla_{AmpC} expression (*ampC/rpsL* ratio of 5.8), whereas isolate ARC3610 had the lowest level of bla_{AmpC} expression when compared with *rpsL* (*ampC/rpsL* ratio of 1.2). The standard deviation of the *ampC/rpsL* ratio of replicate experiments was <0.2. As expected, the ceftazidime-susceptible *P. aeruginosa* isolate ATCC 27853, which was included as a control, had a very low level of bla_{AmpC} expression.

Frequency of spontaneous resistance development to ceftazidime/avibactam

Agar plates containing drug concentrations at multiples of the agar dilution MIC, where avibactam was kept at a fixed concentration of 4 mg/L, were used to determine the frequency of spontaneous resistance which was calculated based on the number of colonies that appeared after 24 h of incubation on the selective plates. The rates were low for all three *P. aeruginosa* isolates and ranged from 2.5×10^{-8} to 1.2×10^{-9} at 4-fold the agar dilution MIC and from 4.4×10^{-9} to $<5.8\times10^{-10}$ at 8-fold the agar dilution MIC (Table 1). A single variant of *P. aeruginosa* ARC3608 was isolated at 16-fold the agar dilution MIC, with a frequency of 6.7×10^{-10}, although three additional colonies that emerged after 48 h of incubation were also characterized (Table 2; strains 3608-64.1, 3608-64.2 and 3608-64.3). No colonies were obtained from the other isolates at this concentration. Additionally, the resistant variants isolated from ARC3608 at 8-fold the agar dilution MIC failed to regrow when restreaked onto selection plates.

Representative resistant variants were selected and checked by broth microdilution for stable changes in MIC values compared with the parent strain. All variants from *P. aeruginosa* ARC3509, irrespective of whether they were selected from the 4-fold MIC or the 8-fold MIC selection plate, resulted in elevated ceftazidime/avibactam MIC values that were 64 mg/L ($n=5$) or 128 mg/L ($n=1$), which represented a 16- or 32-fold increase over the parent strain, respectively (Table 2). Two of these variants, one from each selection concentration, also resulted in increased ceftazidime MIC values from 128 to >256 mg/L. The five variants tested from *P. aeruginosa* ARC3608 had very similar susceptibility profiles across all selection concentrations and incubation periods, where the ceftazidime/avibactam MIC was elevated 16–32-fold compared with the parent strain while the ceftazidime MIC was elevated from 128 to >256 mg/L for four of the five variants (Table 2). Finally, the two variants obtained from *P. aeruginosa* ARC3610 on the 4-fold MIC selection plate demonstrated an 8- and 32-fold elevation of the ceftazidime/avibactam MIC values over the parent strain with ≥4-fold higher ceftazidime MIC values.

We further investigated the susceptibility profiles of the resistant strains towards other drugs (Table 2). These remained unchanged for the non-β-lactam drugs (amikacin, colistin and levofloxacin) between the parent isolates and the resistant variants. However, the susceptibility profiles of other β-lactam drugs were affected. The parent *P. aeruginosa* strain ARC3509 was resistant to the three carbapenems tested, but interestingly the six daughter strains became more susceptible to meropenem, doripenem and imipenem by 4-, 8- and 32-fold, respectively.

Table 1. Frequency of spontaneous resistance to ceftazidime/avibactam in representative *P. aeruginosa* strains

Strain	CAZ/AVI agar dilution MIC (mg/L)	Fold CAZ/AVI MIC[a]	Number of colonies	Frequency of CAZ/AVI resistance
P. aeruginosa ARC3509	4	2×	TNTC	ND[b]
		4×	35	2.5×10^{-8}
		8×	6	4.4×10^{-9}
		16×	0	$<7.3\times10^{-10}$
P. aeruginosa ARC3608	4	2×	TNTC	ND[b]
		4×	15	1.0×10^{-8}
		8×	2[c]	1.3×10^{-9}
		16×	1 (4[d])	6.7×10^{-10}
P. aeruginosa ARC3610	4	2×	TNTC	ND[b]
		4×	2	1.2×10^{-9}
		8×	0	$<5.8\times10^{-10}$
		16×	0	$<5.8\times10^{-10}$

CAZ, ceftazidime; AVI, avibactam; TNTC, too numerous to count.
[a]These concentrations represent multiples of the agar dilution MIC; avibactam held at a constant 4 mg/L.
[b]Not determined due to growth pattern on plates (too many colonies to count).
[c]Neither of these colonies grew when plated onto selective plates (ceftazidime/avibactam at 32/4 mg/L).
[d]Three additional colonies emerged after a further 24 h of incubation (48 h in total).

Table 2. Susceptibility profiles and AmpC status of *P. aeruginosa* strains

	Broth microdilution MIC (mg/L)												
Strain	CAZ	CAZ/AVI[a]	ATM	ATM/AVI[a]	MEM	IPM	DOR	FEP	TZP	AMK	CST	LVX	AmpC sequence[b]
ATCC 27853	2	1	4	4	0.5	2	0.5	2	8	2	0.5	1	PDC-5
ARC3509	128	4	128	16	16	32	16	32	256	8	0.25	4	PDC-8
3509-16A	128	64	32	32	4	1	2	16	32	8	0.25	4	7Δ deletion
3509-16C	>256	64	32	32	4	1	2	16	32	8	0.25	4	7Δ deletion
3509-16D	128	64	32	32	4	1	2	16	32	8	0.25	4	7Δ deletion
3509-32A	128	64	32	32	4	1	2	16	32	8	0.25	4	7Δ deletion
3509-32B	128	64	32	32	4	1	2	16	32	8	0.25	4	7Δ deletion
3509-32C	>256	128	32	32	4	1	2	16	32	8	0.125	2	7Δ deletion
3608	128	8	64	16	32	32	16	32	256	32	1	8	PDC-39
3608-16	>256	256	128	64	32	32	16	32	256	32	1	8	7Δ deletion
3608-64	>256	128	128	32	32	16	8	32	128	32	1	8	7Δ deletion
3608-64.1[c]	>256	128	128	64	32	32	16	32	256	32	1	8	7Δ deletion
3608-64.2[c]	>256	128	128	32	32	32	16	32	256	32	1	8	7Δ deletion
3608-64.3[c]	128	128	64	16	32	32	16	16	256	32	1	8	G183D substitution
3610	64	8	64	16	8	32	8	16	128	32	0.5	16	PDC-37
3610-16A	256	64	32	16	8	2	2	16	32	32	0.5	16	19Δ deletion
3610-16B	>256	256	128	64	8	2	4	32	32	32	0.5	16	7Δ deletion
3610-C1[d]	256	256	256	64	8	4	4	64	64	32	0.5	16	5Δ deletion
3610-C5[d]	256	256	256	64	8	2	4	>64	64	32	0.5	16	5Δ deletion

CAZ, ceftazidime; AVI, avibactam; ATM, aztreonam; MEM, meropenem; IPM, imipenem; DOR, doripenem; FEP, cefepime; TZP, piperacillin/tazobactam; AMK, amikacin; CST, colistin; LVX, levofloxacin.
[a]Avibactam held at a constant concentration of 4 mg/L.
[b]The nomenclature defines the AmpC sequence in the parental strain. PDC-5, PDC-8, PDC-37 and PDC-39 have GenBank accession numbers of ACQ82810, ACQ828130, KJ949052 and KJ949054, respectively.
[c]These mutants emerged after an additional 24 h of incubation.
[d]These two mutants were selected from the *in vitro* PK/PD hollow-fibre experiment.[15]

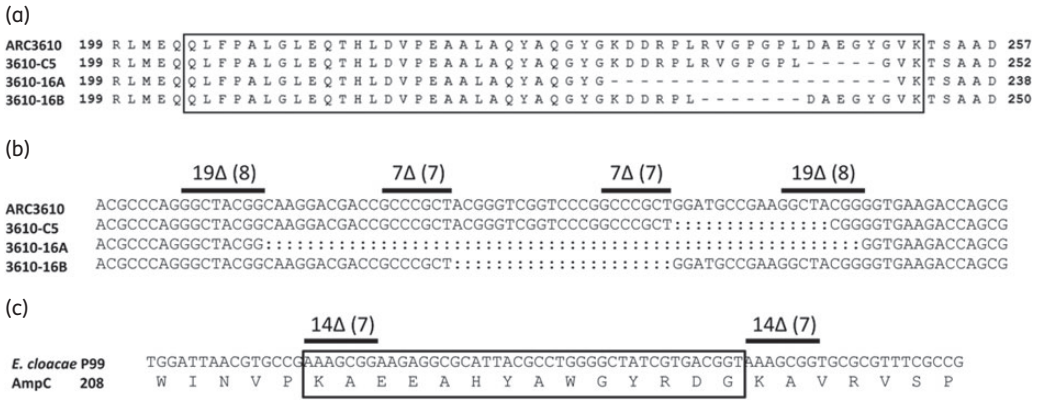

Figure 1. Characterization of Ω-loop mutants. (a) Alignment of the AmpC Ω-loop region of WT isolate ARC3610 and the three different deletion mutants: 3610-C5 isolated from the *in vitro* hollow-fibre cartridge and 3610-16A and 3610-16B isolated from the direct selection experiment. The Ω-loop region is boxed and the deleted residues indicated by a '-'. (b) DNA sequence alignment of the *ampC* allele surrounding the deleted regions. The 8 and 7 bp direct repeats that flank the 19 and 7 residue deletions, respectively, are marked. (c) DNA and amino acid translation of AmpC of *E. cloacae* P99. The 14 amino acid residue deletion reported by Livermore *et al*.[14] is boxed and the 7 bp direct repeats that flank the deletion are marked.

Furthermore, these strains also exhibited 8- and 4-fold reductions in the MIC values of piperacillin/tazobactam and aztreonam, respectively. Similar trends of increased susceptibility to doripenem, imipenem and piperacillin/tazobactam were observed between the parent *P. aeruginosa* ARC3610 and the corresponding mutants. In this case, the meropenem MIC value remained unchanged at 8 mg/L. In contrast, however, *P. aeruginosa* ARC3608 and all its five mutants remained resistant to all β-lactam drugs tested, suggesting that ARC3608 contains other mechanisms of resistance.

In vitro PK/PD and isolation of ceftazidime/avibactam-resistant variant

A hollow-fibre *in vitro* PK/PD experiment using ARC3610 and simulated human exposures of ceftazidime and avibactam has been described previously.[15] However, the growth response of ARC3610 in duplicate cartridges following exposure to ceftazidime/avibactam showed an initial decrease followed by strong regrowth after ∼10 h.[15]

Isolates from both cartridges at the 24 h timepoint contained a ceftazidime/avibactam-resistant population. Representative isolates from both cartridges (3610-C1 and 3610-C5) were included in this study and had identical susceptibility patterns, generating a ceftazidime/avibactam MIC of 256 mg/L, which represented a 32-fold increase over the parent isolate ARC3610 (Table 2). These isolates were included in the follow-up characterization.

Genetic characterization of the *P. aeruginosa* ceftazidime/avibactam-resistant isolates

WGS was used to characterize the variants that were isolated from both the direct selection experiment as well as the hollow-fibre experiment that displayed decreased susceptibility to ceftazidime/avibactam compared with the parental isolates. All of the resistant variants were found to have mutations in the *ampC* coding region that resulted in changes in the AmpC protein sequence. The representative resistant variants from both *in vitro* hollow-fibre cartridges were found to have the same mutation in the bla_{AmpC} allele, which resulted in a five amino acid residue deletion (5Δ) of Asp245, Ala246, Glu247, Gly248 and Tyr249 from the Ω-loop region of the protein (Table 2 and Figure 1a). In contrast, the six resistant variants isolated from the *in vitro* selection experiments from *P. aeruginosa* ARC3509 all carried the same mutation in bla_{AmpC}, which encoded an AmpC protein with a seven residue deletion (7Δ) that removed Arg238, Val239, Gly240, Pro241, Gly242, Pro243 and Leu244, also in the Ω-loop region but immediately proximal to the 5Δ change (Table 2 and Figure 1a). The identical 7Δ change was also present in four of the five resistant variants from isolate ARC3608 as well as one from isolate ARC3610 (Table 2). The whole-genome comparison between parent and daughters did not identify any other genotypic variations that contributed to the susceptibility phenotype of the resistant variants.

In addition to these two deletions observed in this study, there were two additional AmpC variations observed amongst the resistant strains obtained from the *in vitro* selection experiment. First, in variant 3610-16A there was a 19 residue deletion (19Δ) from residues 232 to 250, which extended the 7Δ on either side and fully encompassed the 5Δ (Figure 1a). Interestingly, 19Δ had a smaller elevation of the ceftazidime/avibactam MIC value than either the 5Δ or 7Δ deletion isolated in the same host background (Table 2). Finally, in variant 3608-64.3 there was a single amino acid substitution, Gly183Asp, which is not located in the Ω-loop region (Table 2). This substitution, unlike the Ω-loop deletions, did not result in an elevation of the ceftazidime MIC value, yet resulted in a 16-fold elevation of the ceftazidime/avibactam MIC value.

Examination of the nucleotide sequence of the Ω-loop deletions revealed that the 21 bp region that encodes the 7Δ protein was flanked by a set of 7 bp direct repeats. Similarly, the 57 bp 19Δ modification was also flanked by a set of 8 bp direct repeats (Figure 1b). However, no direct repeats could be detected that flank the 15 bp 5Δ detected in the two mutants isolated from the hollow-fibre system.

Biochemical characterization of the mutant AmpC β-lactamase enzyme

In order to investigate the interplay between the factors of ceftazidime hydrolysis and avibactam inhibition, enzyme kinetic

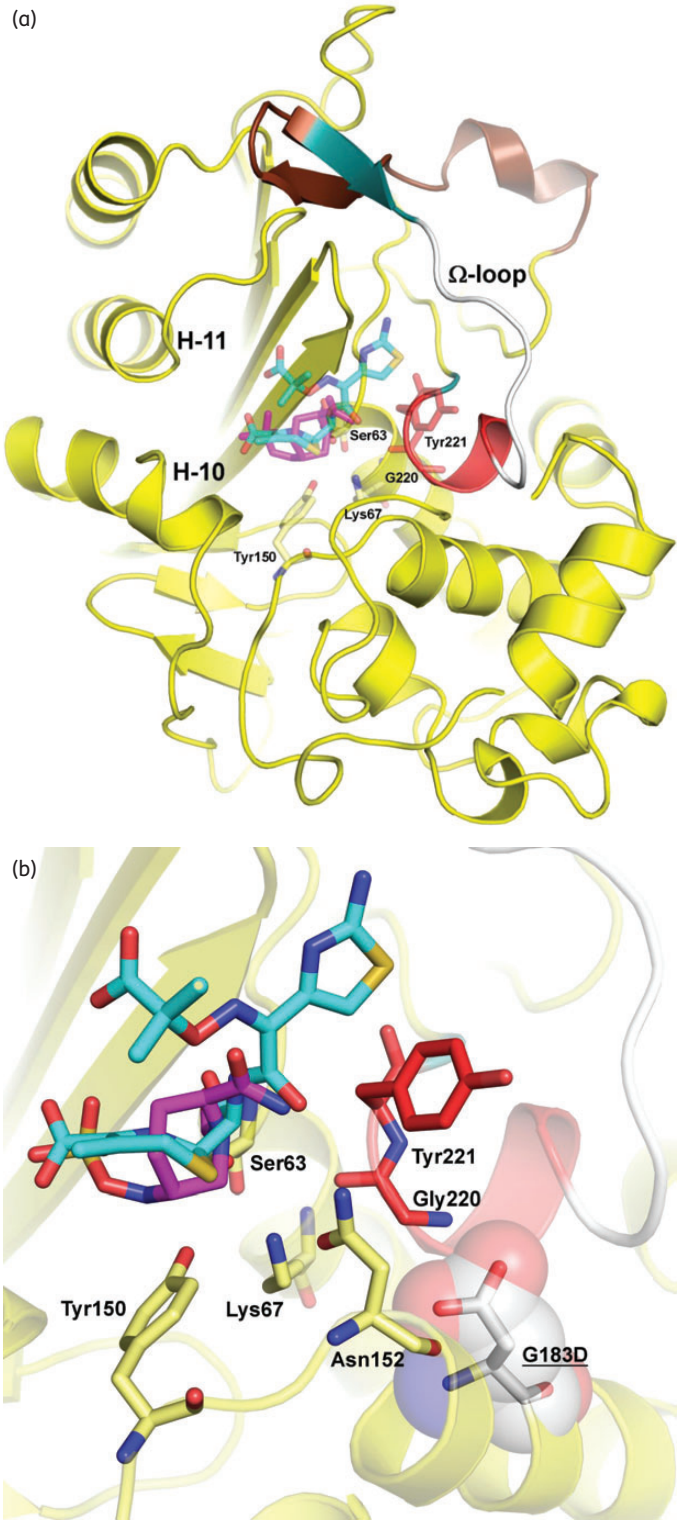

Figure 2. *P. aeruginosa* AmpC residues mutated in the resistant variants. (a) Location of Ω-loop deletions. Avibactam is depicted as pink sticks and ceftazidime is depicted as cyan sticks. The catalytic residues in the binding pocket are depicted as yellow sticks and the residue in the binding pocket that is contributed by the Ω-loop (Tyr221) is depicted as red sticks. The protein is shown as a yellow ribbon; the 5Δ deletion is drawn in red, the 7Δ deletion is drawn in white, the flanking residues of the 19Δ deletion (which includes the 5Δ and 7Δ deletions) are drawn in cyan and the 14 amino acid residue deletion reported by Livermore *et al.*[14] is drawn in brown. (b) Location of the Gly183Asp mutation. Avibactam is depicted as magenta sticks and ceftazidime is depicted as cyan sticks. The residues interacting with these molecules are indicated; the catalytic residues are depicted as yellow sticks, the Ω-loop residues are depicted as red sticks and the Gly183Asp change is depicted as grey sticks (with spheres).

studies were performed for representative AmpC variants. The AmpC enzyme from the clinical isolate ARC3610 (which was used in the hollow-fibre study) as well as the resulting enzyme from the isolate ARC3510-C5 were studied, as was the enzyme from the reference strain PAO1. The ARC3610-C5 enzyme, which contained the 5Δ deletion, gave a significant elevation in the ceftazidime/avibactam MIC comparable to the 7Δ mutants. Importantly, Gly248 and Tyr249 (Gly220 and Tyr221 in class C numbering scheme) that directly form a part of the binding pocket via its backbone atoms (Figure 2a) is an element of the deletion (Figure 1).

The k_{cat} and K_m values and the k_{cat}/K_m ratio were determined for penicillin G and ceftazidime with the three β-lactamase enzymes (AmpC-PAO1, AmpC-3610 and AmpC-3610-C5) and are presented in Table 3. For ceftazidime, the enzymes AmpC-PAO1 and AmpC-3610 showed similar k_{cat}, K_m and overall catalytic efficiency values (k_{cat}/K_m). Ceftazidime was relatively inefficiently hydrolysed by the WT enzymes AmpC-PAO1 and AmpC-3610. In contrast, the mutant enzyme AmpC-3610-C5 showed a 650-fold increase in k_{cat} relative to AmpC-3610, indicating a greater maximal rate of hydrolysis. This was partially offset by an increase in K_m of ∼8-fold. The net change in k_{cat}/K_m for ceftazidime for AmpC-3610-C5 was ∼75-fold greater than for AmpC-3610. Penicillin G was efficiently hydrolysed by both AmpC-PAO1 and AmpC-3610, with very similar results obtained for both k_{cat} and K_m for these two enzymes. The β-lactamase AmpC-3610-C5 was notably less effective at hydrolysing penicillin G, with ∼20-fold reduction in k_{cat} and ∼4-fold increase in K_m as compared with AmpC-3610. Together, these factors resulted in an overall 100-fold decrease in catalytic efficiency as judged by k_{cat}/K_m. A similar change in substrate hydrolysis has previously been documented for AmpC enzymes carrying amino acid substitutions in the Ω-loop,[19,20] although biochemical characterization of an AmpC enzyme carrying an Ω-loop deletion has not been reported to date.

To understand the impact on avibactam inhibition properties, IC_{50} values for avibactam were collected using a standard 5 min pre-incubation with each of AmpC-PAO1, AmpC-3610 and AmpC-3610-C5. By this measure, the potency of avibactam is not significantly different between the WT enzymes AmpC-PAO1 and AmpC-3610 with IC_{50} values of 0.56 ± 0.16 and 0.42 ± 0.17 μM, respectively, expressed as mean ± SD. In contrast, however, avibactam showed a significant decrease in potency against the enzyme AmpC-3610-C5 with an IC_{50} value of 10.65 ± 0.48 μM.

Discussion

In this study, we interrogated *P. aeruginosa* strains that are resistant to ceftazidime and exhibit various levels of chromosomal

Table 3. Kinetic constants for penicillin G and ceftazidime[a]

Enzyme	Substrate	k_{cat} (s^{-1})	K_m[b] (μM)	k_{cat}/K_m (μM^{-1} s^{-1})
AmpC-PAO1	penicillin G	56	21	2.7
AmpC-3610	penicillin G	50	30	1.7
AmpC-3610-C5	penicillin G	2.3	130	0.017
AmpC-PAO1	ceftazidime	0.007	60	0.0001
AmpC-3610	ceftazidime	0.006	150	0.00004
AmpC-3610-C5	ceftazidime	3.9	1210	0.003

[a]For clarity, standard errors of means have been omitted. All results were calculated as the means from a minimum of triplicate tests. For k_{cat} and K_m, the standard error of the mean was <15%.
[b]For ceftazidime, K_m was determined as the K_i in competition with nitrocefin.

AmpC β-lactamase expression, to determine whether these differences influence the ability of these strains to develop resistance to ceftazidime/avibactam. The class C enzymes, unlike many of the class A and some of the class D enzymes, are recalcitrant to inhibition by the clinically used β-lactamase inhibitors such as sulbactam and tazobactam. Therefore, emergence of clinical resistance to the newer cephalosporins, such as ceftazidime and cefepime, in pathogens carrying class C enzymes has selected for improved substrate hydrolysis properties rather than the selective pressure to avoid inhibition, as in the case of class A inhibitor-resistant TEM enzymes. An increased expression of these enzymes is a common mechanism whereby the bacterial cell is able to increase drug hydrolysis and render these strains resistant. Avibactam is a potent inhibitor of class C β-lactamases that has previously been shown to maintain its effectiveness against strains containing highly derepressed chromosomal *ampC* gene.[4,13] This inhibition enabled the addition of avibactam to ceftazidime to revert the MIC distribution for an unselected population of clinical isolates of *P. aeruginosa* to approximately that of the 'WT' distribution of ceftazidime MICs.[21] Avibactam interacts primarily with the conserved residues that have been implicated in β-lactam catalysis and as such is able to inhibit a broad spectrum of β-lactamases despite the variability in both sequence and structure that these enzymes possess.[13] The effectiveness of the ceftazidime/avibactam combination in a highly derepressed *ampC* gene background was evident in this study, where the frequency of spontaneous resistance to the ceftazidime/avibactam combination in all three strains was very low. Additionally, these numbers were several orders of magnitude lower than with either imipenem or meropenem,[22] suggesting that the risk of developing resistance spontaneously to this drug combination during therapy is low. In studies of simulated human exposures of ceftazidime/avibactam against *P. aeruginosa* infections in neutropenic and immunocompetent mice, no resistant variants were observed on post-therapy drug selection plates.[15,23]

The few resistant colonies that were observed in this experiment served as tools to understand the molecular changes that could result in resistance against such a combination. Interestingly, all the changes observed in this study were in AmpC, the vast majority of which were in the Ω-loop region. The Ω-loop of AmpC has previously been noted to significantly impact the catalytic efficiency and spectrum of cephalosporin hydrolysis and the enzymes possessing changes within this region are broadly referred to as 'extended-spectrum AmpC' (ESAC) β-lactamases.[24–26] Structural information of AmpC bound to both ceftazidime and avibactam shows that the Ω-loop region interacts with the bulky oxyimino group of ceftazidime, which sterically prevents the drug from adopting an optimal conformation for hydrolysis unlike the earlier generations of cephalosporins.[26] Two residues of the Ω-loop, Gly220 and Tyr221 (of the 5Δ 'DAEGY' deletion), also form a part of the avibactam-binding pocket, where Tyr221 provides a hydrophobic surface and Gly220 interacts with the catalytic residue, Lys67, which is expected to be important for inhibition (Figure 2a and b).[13] Taken together, the changes in the Ω-loop observed here are expected to influence both ceftazidime hydrolysis and avibactam inhibition. The deletions resulting in a truncated Ω-loop are expected to widen this part of the binding pocket by disrupting and opening up a helix (Figure 2a, in red). This structural change would increase ceftazidime binding and subsequent hydrolysis by removing the steric hindrance and better accommodating the oxyimino group (Figure 2a and b),[27] which is consistent with the observed increase in ceftazidime hydrolysis efficiency. In contrast, removal of the steric hindrance may allow the carbapenems to rotate their bulky 6α-hydroxyethyl side chains and displace the point of hydrolytic attack more effectively, thus explaining the increased carbapenem susceptibility.[27,28] The impact on avibactam inhibition appears to be the result of deletion or misplacement of Gly220 and Tyr221 that is likely to reduce the acylation rate of the inhibitor.

The single residue mutation observed in this study (Gly183Asp) that is outside of the Ω-loop region is intriguing. This change is located in the second sphere of the residues that form the binding pocket and is spatially close to Asn152, a critical residue for both β-lactam catalysis and avibactam binding[13] (Figure 2b). A mutation from Gly to Asp results in a significantly larger and negatively charged group in this region that can easily hamper avibactam binding. Gly183 is also in close contact with the Ω-loop residues Gly220 and Tyr221 and thus can alter the position of these latter residues. Although we did not observe an elevation of the ceftazidime MIC for the isolates containing the Gly183Asp mutation, this mutation has recently been observed to cause a 32-fold decrease in *P. aeruginosa* susceptibility to ceftolozane during a serial passage experiment,[29] suggesting that this change also represents an ESAC phenotype to more recent cephalosporins.

Previous studies with other naturally occurring ESAC variants have shown that nearly all of the enzymes that contained these changes in the H-10 and H-11 (Figure 2a) helix are inhibited effectively by avibactam.[30] There was only a single rare observation of a six amino acid deletion in the H-10 helix that reduced, but did not abolish, the inhibitory property of avibactam.[31] However, the impact of Ω-loop changes on avibactam inhibition has been less characterized. In our experiments, we observed all changes in or near the Ω-loop that resulted in decreased avibactam inhibition were concomitant with an increased hydrolysis of the β-lactam ceftazidime. The preference for these deletions can be explained by the presence of short direct repeats that flank the deleted residues and can result in looping out of the intervening DNA. The only other Ω-loop change previously reported for an avibactam combination was in AmpC isolated from *Enterobacter cloacae* selected in the presence of ceftaroline/avibactam, in which a 14 amino acid deletion had occurred immediately proximal to that observed in this study (Figure 1c and Figure 2a).[14,32] Interestingly, this deletion

in *E. cloacae* AmpC was also flanked by 7 bp direct repeats, suggesting that a similar mechanism contributed to the Ω-loop deletions in both species.

Overall, the frequency of resistance development in *P. aeruginosa* exhibiting derepressed *ampC* alleles to the combination of ceftazidime and avibactam was low. The few resistant mutants isolated all had changes in the AmpC β-lactamase with the great majority being small deletions in the Ω-loop that impacted both ceftazidime hydrolysis and avibactam inhibition. This is different from the point mutations previously observed in the resistant strains selected on the aztreonam/avibactam combination, which were functionally conservative substitutions localized to the catalytic and binding residues.[13] Changes in the Ω-loop with avibactam have only been previously described in an *E. cloacae* resistant mutant after selection on a combination of ceftaroline and avibactam.[14] Interestingly, all Ω-loop changes increased the susceptibility to carbapenems. Together, this suggests that resistance to avibactam is influenced by the partner β-lactam and that the mechanisms of resistance to avibactam in combination with cephalosporins may differ from that with other β-lactams. Additionally, resistance appears to be detectable *in vitro* but no resistance has been seen thus far in either *in vivo* studies or in the clinical programme. The likely explanation is that the clinical dose suppresses development of most mutants. Thus, the combination of ceftazidime and avibactam should continue to be developed as a promising therapeutic alternative to treat infections caused by drug-resistant *P. aeruginosa*.

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