J Antimicrob Chemother 2017; **72**: 1400–1409
doi:10.1093/jac/dkw587 Advance Access publication 30 January 2017

Journal of Antimicrobial Chemotherapy

Pharmacodynamics of ceftazidime/avibactam against extracellular and intracellular forms of *Pseudomonas aeruginosa*

J. M. Buyck[1]†, C. Luyckx[1], G. G. Muccioli[2,3], K. M. Krause[4]‡, W. W. Nichols[5], P. M. Tulkens[1] and F. Van Bambeke[1]*

[1]*Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium;* [2]*MASSMET Platform, Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium;* [3]*Bioanalysis and Pharmacology of Bioactive Lipids Research Group, Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium;* [4]*Cerexa Inc., Oakland, CA, USA;* [5]*AstraZeneca Pharmaceuticals, Waltham, MA, USA*

*Corresponding author. Tel: +32-2-764-73-78; E-mail: francoise.vanbambeke@uclouvain.be
†Present address: UFR Médecine et Pharmacie, INSERM U1070, Université de Poitiers, Poitiers, France.
‡Present address: Achaogen Inc., South San Francisco, CA, USA.

Received 13 October 2016; returned 24 November 2016; revised 13 December 2016; accepted 20 December 2016

Objectives: When tested in broth, avibactam reverses ceftazidime resistance in many *Pseudomonas aeruginosa* that express ESBLs. We examined whether similar reversal is observed against intracellular forms of *P. aeruginosa*.

Methods: Strains: reference strains; two engineered strains with basal non-inducible expression of AmpC and their isogenic mutants with stably derepressed AmpC; and clinical isolates with complete, partial or no resistance to reversion with avibactam. Pharmacodynamic model: 24 h concentration–response to ceftazidime [0.01–200 mg/L alone or with avibactam (4 mg/L)] of bacteria in broth or bacteria phagocytosed by THP-1 monocytes, with calculation of ceftazidime relative potency (C_s: concentration yielding a static effect) and maximal relative effect [E_{max}: cfu decrease at infinitely large antibiotic concentrations (efficacy in the model)] using the Hill equation. Cellular content of avibactam: quantification by LC-MS/MS.

Results: For both extracellular and intracellular bacteria, ceftazidime C_s was always close to its MIC. For ceftazidime-resistant strains, avibactam addition shifted ceftazidime C_s to values close to the MIC of the combination in broth. E_{max} was systematically below the detection limit (-5 $\log_{10}$) for extracellular bacteria, but limited to -1.3 $\log_{10}$ for intracellular bacteria (except for two isolates) with no effect of avibactam. The cellular concentration of avibactam reflected extracellular concentration and was not influenced by ceftazidime (0–160 mg/L).

Conclusions: The potential for avibactam to inhibit β-lactamases does not differ for extracellular and intracellular forms of *P. aeruginosa*, denoting an unhindered access to its target in both situations. The loss of maximal relative efficacy of ceftazidime against intracellular *P. aeruginosa* was unrelated to resistance via avibactam-inhibitable β-lactamases.

Introduction

Pseudomonas aeruginosa, a major cause of nosocomial infections in immunocompromised or debilitated patients, is of concern to clinicians because of a high level of resistance in contemporary isolates through an array of mechanisms, among which constitutive and inducible expression of β-lactamases (including ESBLs and carbapenemases) play an important role.[1] *P. aeruginosa* is also able to enter, survive and even thrive in eukaryotic cells where the efficacy of most antibiotics is considerably reduced compared with what is observed against extracellular bacteria when tested in appropriate pharmacodynamic models.[2]

Avibactam (formerly AVE1330A[3] and NXL104;[4,5] see the recent review by Wang *et al.*[6]) is a non-β-lactam ESBL inhibitor with activity against most class A and class C β-lactamases as well as some class D enzymes.[7,8] In broth, avibactam fully reverses AmpC- and ESBL PER-1-mediated ceftazidime resistance in *P. aeruginosa*,[9,10] which translates to restoration of ceftazidime against this organism in wide-scale surveillance studies.[11–13] These results also show that avibactam reaches the bacterial periplasm and, therefore, crosses the outer membrane of *P. aeruginosa*.

Ceftazidime/avibactam has been approved in the USA for the treatment of complicated intra-abdominal infections (cIAI) (in combination with metronidazole) and also for the treatment of complicated urinary tract infections (cUTI) in patients with limited or no alternative treatment options.[14] It has also been

approved by the EMA for the treatment of adults with cIAI, cUTI (including pyelonephritis) or hospital-acquired pneumonia (including ventilator-associated pneumonia), and for the treatment of infections due to aerobic Gram-negative organisms in patients with limited treatment options.[15]

Our aim was to examine whether avibactam restores ceftazidime activity against intracellular forms of β-lactamase-producing *P. aeruginosa*, which entails not only crossing the bacterial outer membrane but also the eukaryotic pericellular membrane and those of the intracellular vacuoles hosting the bacteria.[16] To this effect, we used a pharmacodynamic model originally developed in our laboratory for the quantitative study of the intracellular activity of antibiotics against phagocytosed *Staphylococcus aureus*[17] and validated for similar studies with *P. aeruginosa*.[16] We show here that, when tested against intracellular forms of *P. aeruginosa* that produce avibactam-inhibitable β-lactamase(s), avibactam restores ceftazidime activity to the same extent as in broth.

Materials and methods

Bacterial strains, susceptibility testing and genotypic detection of β-lactamases

The panel of strains assembled is shown in Table 1. Two engineered strains with a basal non-inducible level of expression of AmpC (M1405 def and 2297 def) and their corresponding spontaneous mutants with stably derepressed constitutive hyperproducers of AmpC (M1405 CON and 2297 CON) were from Professor D. Livermore,[18,19] and the clinical isolates with variable levels of susceptibility to ceftazidime from Belgian teaching hospitals.[20] Bacteria were grown in Mueller–Hinton broth and cfu counting was performed by plating serial dilutions on tryptic soy agar. MICs were measured according to the 2014 CLSI guidelines.[21] Detection of genes encoding known β-lactamases (see list in Table 1) was performed using a set of three multiplex endpoint PCR assays using appropriate primers.[22]

Materials

Avibactam sodium (potency 91.7%) and AZ13466915 were provided by AstraZeneca Pharmaceuticals (Waltham, MA, USA and Alderly Park, UK, respectively). Ceftazidime was obtained as Glazidim® and gentamicin as Gentalline®, the corresponding branded products for human parenteral use in Belgium and complying with the provisions of the European Pharmacopoeia. Colistin [sulphate salt (potency 67.5%)] was from Sigma-Aldrich, St Louis, MO, USA, levofloxacin (potency 95%) was from Aventis Pharma, Bad Soden, Germany and tobramycin [base (potency 95.8%)] was from Teva, Wilrijk, Belgium. Human serum was from Biowest SAS, Nuaillé, France, and cell culture media and FCS were from Gibco/Life Technologies Corporation (Paisley, UK). All other products were obtained from Sigma-Aldrich or Merck KGaA (Darmstadt, Germany).

Cells, cell culture and intracellular infection

Human THP-1 monocytes were cultivated as previously described[17] and intracellular infection was performed following a published protocol.[2,16] In brief, bacteria were opsonized with 10% human serum in RPMI-1640 medium, phagocytosis was allowed for 2 h at a bacterium:cell ratio of 10:1 and non-phagocytosed bacteria were eliminated by incubation with gentamicin (100 mg/L, 60 min, 37°C) and three washes in PBS. The intracellular inoculum was typically 5–7 × 10^5 cfu/mg of cell protein.

Table 1. MICs of ceftazidime alone (CAZ) or of ceftazidime combined with a fixed concentration of avibactam (4 mg/L; CAZ/AVI)

	MIC (mg/L)		
Strain	CAZ	CAZ/AVI	Note(s) and β-lactamase detection
Reference strains			
ATCC 27853	2	2	reference strain
PAO1	8	2	
Engineered strains			
2297	2	2	
2297 def	2	2	AmpC-negative derivative of 2297
2297 CON	128	8	AmpC-positive derivative of 2297[a]
M1405 def	4	4	AmpC-negative derivative of M1405[b]
M1405 CON	128	8	AmpC-positive derivative of M1405[a,b]
Clinical isolates			
PA67	4	1	[c]
PA112A	8	8	[c]
PA128	2	1	[c]
PA129	2	2	[c]
PA166	1	1	[c]
PA196	8	4	[c]
PA229	4	2	[c]
PA302	2	2	[c]
PA344	1	1	[c]
PA348	8	4	[c]
PA356	8	8	[c]
PA358	8	8	[c]
PA27	64	4	[c]
PA59	64	4	[c,d]
PA65	16	2	[c]
PA94A	64	8	[c]
PA104	64	4	[c,d]
PA115	64	4	[c]
PA119	128	16	[c]
PA139	32	8	[c]
PA152	128	4	[c,d]
PA156	128	4	[c,d]
PA185	64	8	[c]
PA281	16	4	[c]
PA299	256	8	[c,d]
PA315	128	4	[c,d]
PA327	16	1	[c]
PA331	256	8	[c,d]
PA340	128	4	[c,d]
PA362	64	4	[c]
PA133	64	32	[c]
PA240	32	32	VIM-2 positive
PA242	32	32	VIM-2 positive
PA254	32	32	VIM-2 positive
PA256	32	32	VIM-2 positive
PA278	64	64	[c]
PA353	256	256	VIM-2 positive
PA372A	256	64	[c]

Strains in bold are those used for the dose–response studies (pharmacodynamic model) shown in Figures 2–4.
[a]MIC of piperacillin/tazobactam = 256 mg/L.
[b]The parent strain M1405 was not available for testing.
[c]Negative for the following β-lactamases as detected by genomic techniques: OXA-1, CTX-M-1, CTX-M-2, CTX-M-9 group, SHV, TEM, VIM, IMP, NDM and KPC using a set of three multiplex endpoint PCR assays.[22]
[d]MIC of piperacillin/tazobactam >256 mg/L.

Pharmacodynamic studies in broth (extracellular activity) and in cells (intracellular activity)

Ceftazidime was added to the medium [inoculated broth (10^6 cfu/mL) or cell culture medium of infected cells (see above)] at extracellular concentrations ranging from 0.01 to 200 mg/L alone or in combination with a fixed concentration of avibactam (4 mg/L, unless stated otherwise) to obtain a full concentration–response curve to the antibiotic. After 24 h of incubation at 37°C, samples were collected and treated as previously described.[16] In brief, for studies in broth, samples were serially diluted to enable viable counting and to minimize antibiotic carry-over, after which 50 μL of suspension was seeded on tryptic soy agar and colonies counted after 24 h of incubation at 37°C. For intracellular activity studies, cells were pelleted by low-speed centrifugation (1000 rpm, room temperature, 10 min), gently resuspended in PBS at 4°C, pelleted again (1000 rpm, 4°C, 10 min) to fully eliminate extracellular bacteria and minimize antibiotic carry-over, and resuspended in distilled water. After dilution, cell lysates were used for cfu counting by plating on tryptic soy agar and for measurement of total protein content by Lowry's assay (Bio-Rad DCTM Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA). Both extracellular and intracellular activities were expressed as the change of cfu (per mL for studies in broth and per mg cell protein for studies in cells) from the initial inoculum (time 0) in $\log_{10}$ units [ratio of post-treatment cfu to pre-treatment cfu, each expressed per mL (broth) or per mg cell protein (cells)]. Normalization for cell-associated cfu was made with respect to total cell protein rather than to cell numbers because our experience with the model was that a biochemical assay measuring total cell mass yielded more reliable and consistent results across successive experiments and conditions, partly due to the intrinsic variations associated with visual (microscopy) as well as automated cell counting methods.

Cellular penetration of avibactam

To assess the cellular penetration of avibactam, 10^7 THP-1 cells in a volume of 25 mL were incubated at 37°C with avibactam alone or combined with ceftazidime, pelleted (1300 rpm, 4°C, 7 min), washed twice in PBS at 4°C, resuspended in 200 μL of distilled water and sonicated to achieve homogenization (naked eye examination). AZ13466915, closely related to avibactam, was added to the samples at a final concentration of 1 mg/L as internal standard. A calibration curve was obtained from cell lysates spiked with increasing concentrations of avibactam and with 1 mg/L internal standard. Samples (100 μL) were mixed with 750 μL methanol/acetonitrile (4:21, v/v), vortexed for 1 min, kept at −20°C for 30 min and then centrifuged at 2500 rpm for 20 min. The supernatant was collected, dried under nitrogen and dissolved in 100 μL methanol/water (75:25, v/v). Avibactam was quantified by HPLC-MS/MS (Thermo Fisher Scientific Inc., Waltham, MA, USA) using an Accela HPLC [C18 column (150 × 4 mm, pore size 3 μm) with pre-column and an elution gradient of acetonitrile–H_2O (10:90)/acetonitrile (both with 0.1% formic acid to favour ionization)], and an LTQ-Orbitrap [electrospray ionization in negative mode; detection of ions of *m/z* 264.02958 (avibactam) and 302.05645 (AZ13466915) in MS and of *m/z* 96.95996 (both compounds) in MS2]. The avibactam cellular concentration was calculated using a cell volume to protein ratio of 5 μL/mg protein,[17] a value close to that found experimentally for cultured fibroblasts[23] and mouse peritoneal macrophages.[24]

Curve fittings and statistical analyses

Curve fittings were performed with GraphPad Prism (version 7.02) using the Hill equation (sigmoidal dose response) with slope factor set to 1, and statistical analyses with GraphPad InStat 3.10, both for Windows (GraphPad Prism Software, San Diego, CA, USA).

Pharmacodynamic indices

The following pharmacodynamic indices were derived from data obtained from experiments examining concentration–effect relationships and to which the Hill equation could be fitted: E_{min} and E_{max} are the changes in cfu extrapolated for an infinitely low and infinitely large antibiotic concentration, respectively [minimal and maximal pharmacological effects of ceftazidime (minimal and maximal relative antibacterial efficacies in the model)]; C_s is the concentration yielding no apparent change in cfu from the original inoculum [static effect (relative antibacterial potency in the model)].[17]

Results

Susceptibility to ceftazidime and ceftazidime/avibactam

Table 1 shows the MICs of ceftazidime and ceftazidime combined with a fixed concentration of avibactam (4 mg/L) for the reference and engineered strains and the clinical isolates. Based on ceftazidime MICs measured without and with avibactam, strains and isolates were assembled into three groups: group 1, those susceptible to ceftazidime (EUCAST interpretive criteria) for which the addition of avibactam had no effect [ATCC 27854, strains 2297 def and M1405 def and the parent strain 2297, and 12 clinical isolates (PAO1 was susceptible to ceftazidime but showed an MIC decrease from 8 to 2 mg/L with avibactam)]; group 2, those resistant to ceftazidime but made susceptible by addition of avibactam [strains 2297 CON and M1405 CON and 18 clinical isolates (which, when tested, proved also resistant to piperacillin/tazobactam); one isolate (PA119) showed a marked decrease in its MIC with avibactam but was still categorized as resistant based on the EUCAST ceftazidime susceptibility breakpoint]; and group 3, those resistant to ceftazidime and remaining resistant in the presence of avibactam (eight clinical isolates). All strains and isolates were susceptible to gentamicin, tobramycin, levofloxacin and colistin.

To ensure that a 4 mg/L concentration of avibactam was sufficient to fully restore the activity of ceftazidime in the strains and isolates intended for our experiments, we examined the effect of varying its concentration (from 0.03 to 128 mg/L) on ceftazidime MIC, using: (i) most strains and isolates from group 2; and (ii) selected isolates from groups 1 and 3 as controls. The results (see Figure S1, available as Supplementary data at *JAC* Online) showed that the resistance of 12 out of the 18 isolates from group 2 plus the two engineered strains 2297 CON and M1405 CON was fully counteracted with 4 mg/L avibactam (no further decrease in ceftazidime MICs by increasing avibactam concentration). In contrast, 3 isolates from the same group (PA119, PA185, PA331) showed a further decease in ceftazidime MIC when the avibactam concentration was increased to >4 mg/L.

Extracellular and intracellular activity of ceftazidime/avibactam against P. aeruginosa isolates with differing susceptibilities

Full 24 h ceftazidime concentration–response studies (aimed at determining and comparing the pharmacodynamic indices E_{min}, E_{max} and C_s) were performed using ceftazidime alone and ceftazidime combined with avibactam (4 mg/L) for six selected strains: (i) the ATCC 27853 reference strain; (ii) one clinical isolate (PA152) resistant to ceftazidime alone but susceptible when tested with avibactam; and (iii) the two engineered linked parent–daughter (isogenic) pairs M1405 def and 2297 def with basal AmpC

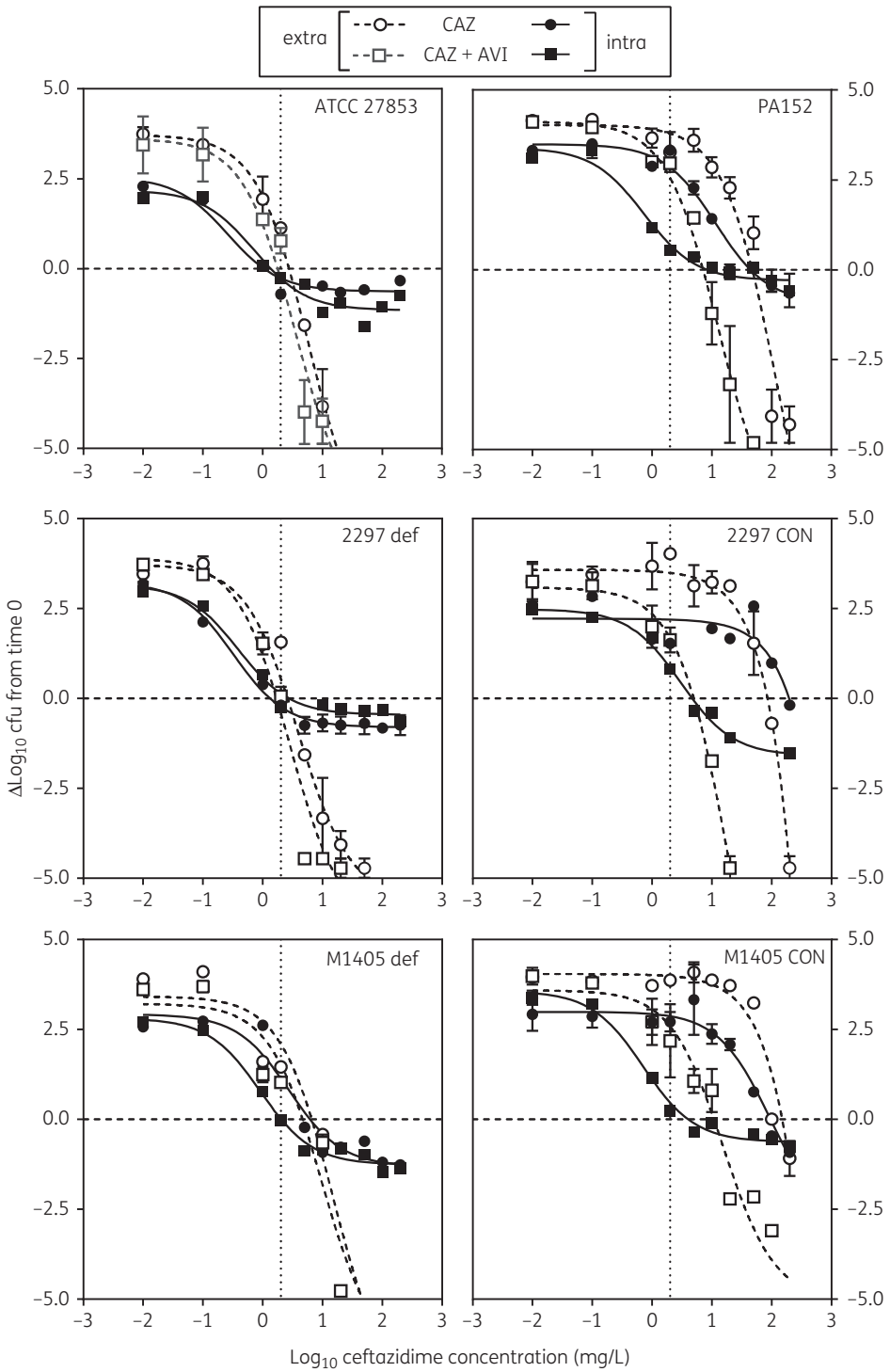

Figure 1. Concentration–response curves of ceftazidime alone (CAZ) and of ceftazidime combined with a fixed concentration (4 mg/L) of avibactam (CAZ + AVI) against the extracellular (extra) and intracellular (intra) forms of *P. aeruginosa* strains (ATCC 27853, reference strain fully susceptible to ceftazidime; and PA152, clinical strain resistant to ceftazidime but susceptible to CAZ + AVI) and the engineered linked parent–daughter (isogenic) pairs with basal non-inducible AmpC (strains M1405 def and 2297 def, low MIC of ceftazidime) and their corresponding spontaneous mutants with stably derepressed AmpC (strains M1405 CON and 2297 CON, high MIC of ceftazidime). The graphs show the change in the number of cfu (Δ $\log_{10}$ cfu from the initial inoculum) per mL of broth (extracellular, open symbols, broken lines) or per mg of cell protein (intracellular, filled symbols, continuous lines) in THP-1 cells after 24 h of incubation at increasing extracellular concentrations of ceftazidime (mg/L; total drug). The limit of detection was −5 $\log_{10}$ cfu from the initial inoculum (time 0 h). The thick broken horizontal line corresponds to a bacteriostatic effect (no apparent change from initial inoculum). The thin broken vertical line indicates the MIC of ceftazidime tested in combination with 4 mg/L avibactam in broth for the strain shown on the graph.

Table 2. Pertinent regression parameters of dose–response curves of ceftazidime alone or of ceftazidime plus a fixed concentration of avibactam (4 mg/L) for extracellular (broth) and intracellular (THP-1 monocytes) activity against selected *P. aeruginosa* strains

		Extracellular activity (broth)				Intracellular activity (THP-1 monocytes)			
				C_s[d]				C_s[d]	
Strain[a]	Avibactam	E_{min}[b]	E_{max}[c]	mg/L	× MIC	E_{min}[b]	E_{max}[c]	mg/L	× MIC
ATCC 27853[e]	–	3.9 ± 0.3	< −5	2.3	1.2	2.5 ± 0.4	−0.6 ± 0.2	1.1	0.6
	+	3.7 ± 0.5	< −5	1.9	1.0	2.2 ± 0.3	−1.2 ± 0.2	1.4	0.7
PA152[f]	–	4.0 ± 0.3	< −5	47.6	0.4	3.5 ± 0.1	−0.9 ± 0.2	44.3	0.4
	+	4.2 ± 0.4	< −5	6.6	1.7	3.4 ± 0.2	−0.3 ± 0.1	8.4	2.1
M1405 def[g]	–	3.6 ± 0.5	< −5	5.6	1.4	2.9 ± 0.4	−1.3 ± 0.3	6.1	1.5
	+	3.4 ± 0.4	< −5	4.1	1.0	2.8 ± 0.2	−1.3 ± 0.1	2.0	0.5
M1405 CON[g]	–	4.0 ± 0.2	< −5	149.7	1.2	3.0 ± 0.2	−2.9 ± 1.4	95.4	0.7
	+	3.6 ± 0.3	< −5	10.8	1.4	3.6 ± 0.1	−0.6 ± 0.1	4.0	0.5
2297 def[g]	–	3.8 ± 0.3	< −5	2.5	0.8	3.2 ± 0.3	−0.8 ± 0.1	1.3	0.4
	+	4.0 ± 0.4	< −5	1.6	0.5	3.2 ± 0.2	−0.5 ± 0.1	2.7	0.9
2297 CON[g]	–	3.6 ± 0.2	< −5	85.4	0.7	2.2 ± 0.3	ND[h]	196.0	1.5
	+	3.4 ± 0.3	< −5	3.4	0.4	2.5 ± 0.2	−1.6 ± 0.2	4.7	0.6

Data are from the experiments shown graphically in Figure 2 and calculated from individual Hill–Langmuir functions (sigmoidal equations with slope factor = 1) fitted to each set of data (strains and conditions).
[a]See Table 1 for ceftazidime MICs in broth with and without avibactam.
[b]cfu increase (in $\log_{10}$ units) at 24 h from the corresponding initial inoculum as extrapolated from infinitely low antibiotic concentration using the Hill–Langmuir equation [= minimal pharmacological effect (minimal relative antibacterial efficacy in the model), corresponding to bacterial growth in the absence of antibiotic].
[c]cfu decrease (in $\log_{10}$ units) at 24 h from the corresponding initial inoculum as extrapolated from infinitely large antibiotic concentration using the Hill–Langmuir equation [= maximal pharmacological effect (maximal relative antibacterial efficacy in the model), corresponding to the maximal bacterial eradication that can be obtained with the antibiotic]; the practical limit of detection in our experiments was -5 $\log_{10}$ cfu from the initial inoculum (time 0 h) and the calculated E_{max} values below this value are indicated as < -5.
[d]Extracellular concentration (in multiples of the MIC; total drug) at which there is no apparent change in cfu compared with the original inoculum, as determined by graphical intrapolation using the Hill–Langmuir equation.
[e]Reference strain.
[f]Ceftazidime-resistant clinical isolates.
[g]AmpC-positive (CON) and AmpC-negative (def) isogenic strains.
[h]Not determined (plateau not reached at the highest concentration tested).

expression (low MIC of ceftazidime) and their corresponding spontaneous mutants with stably derepressed AmpC (strains M1405 CON and 2297 CON; high MIC of ceftazidime). Results are shown in Figure 1 and Table 2.

Considering bacteria grown in broth first, all C_s values were close to the corresponding MICs and all E_{max} values below the actual limit of detection. Addition of avibactam did not change the response of ATCC 27853 to ceftazidime alone. In contrast, the situation drastically changed for PA152 for which the addition of avibactam caused a shift of the concentration-dependent curve to the left (becoming essentially similar to that of ATCC 27853), with: (i) a C_s value close to the ceftazidime MIC for this isolate as measured in the presence of avibactam; and (ii) the lowest limit of detection observed at ceftazidime concentrations much lower than without avibactam and similar to those observed with ATCC 27853.

Moving now to intracellular bacteria, we see first that E_{max} values for all strains were considerably smaller in magnitude (less negative; only -1 to -2 $\log_{10}$ cfu compared with the original inoculum) than for bacteria in broth. E_{max} of lower magnitude for intracellular bacteria compared with bacteria in broth have already been described for other β-lactams when tested with *P. aeruginosa*[16] and *S. aureus* in this model.[17,25] With respect to C_s, the values observed with ceftazidime or ceftazidime combined with avibactam were close to the corresponding MICs, indicating a marked increase in relative potency (lower C_s values) caused by avibactam. In contrast, there was no change in intracellular E_{max} values when combining avibactam with ceftazidime.

Examining now the results obtained with the engineered linked parent–daughter (isogenic) pairs of *P. aeruginosa*, we see that strains 2297 def and M1405 def showed results essentially similar to those seen previously with strain ATCC 27853. Thus, for bacteria in broth, a marked bactericidal effect of ceftazidime, with E_{max} values below the lowest detection level, was achieved together with C_s values close to the MIC of ceftazidime for the corresponding strains in broth. For intracellular bacteria, E_{max} values of a much lower magnitude (i.e. less negative) were obtained but C_s values were still close to the MIC of ceftazidime for the corresponding strains in broth. The addition of avibactam caused no meaningful change in the response of these strains to ceftazidime. For strains

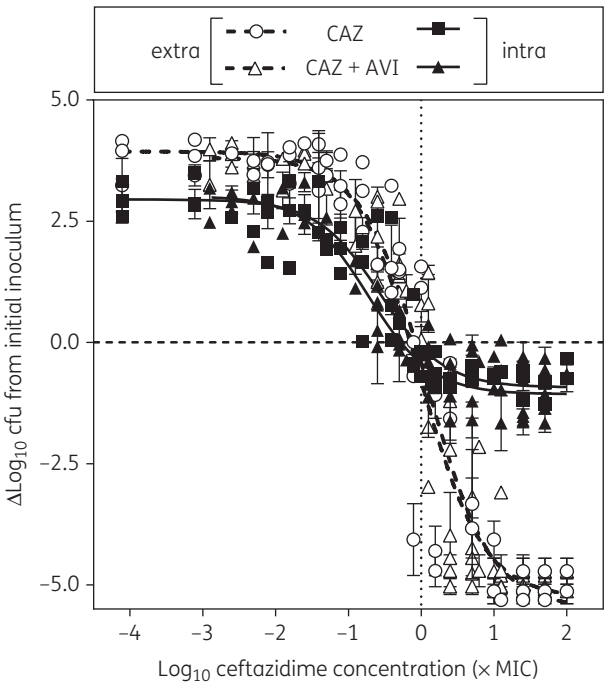

Figure 2. MIC-normalized concentration–response curves of ceftazidime alone (CAZ) and of ceftazidime combined with a fixed concentration (4 mg/L) of avibactam (CAZ + AVI) against the extracellular and intracellular forms of all strains shown in Figure 2 (ATCC 27853, PA152, M1405 def, M1405 CON, 2297 def and 2297 CON). The graphs show the change in the number of cfu (Δ $\log_{10}$ cfu from the initial inoculum) per mL of broth (extracellular, open symbols, broken lines) or per mg of cell protein (intracellular, filled symbols, continuous lines) in THP-1 cells after 24 h of incubation at increasing extracellular concentrations of ceftazidime expressed in $\log_{10}$ of the multiple of its MIC in broth for the corresponding strain in the absence or in the presence of avibactam (ATCC 27853, 2 and 2 mg/L; PA152, 128 and 4 mg/L; M1405 def, 4 and 4 mg/L; M1405 CON, 128 and 8 mg/L; 2297 def, 2 and 2 mg/L; and 2297 CON, 128 and 8 mg/L). The limit of detection was −5 $\log_{10}$ cfu compared with the initial inoculum (time 0 h). The thick broken horizontal line corresponds to a bacteriostatic effect (no apparent change from the initial inoculum). The thin broken vertical line corresponds to 1× the MIC (and is, by definition, common for all strains).

2297 CON and M1405 CON, results were similar to those observed with PA152, i.e.: (i) E_{max} values for bacteria in broth were below the lowest limit of detection, which was reached only at very large concentrations for 2297 CON (for M1405 CON, the extracellular concentration of ceftazidime could not be increased enough to obtain a bactericidal effect) with C_s values close to the ceftazidime MIC; and (ii) a marked shift of the curves to the left upon addition of avibactam, making them essentially similar to those obtained with 2297 def and M1405 def. Avibactam, however, did not change in a meaningful way the E_{max} values of either strain, which, for bacteria in broth, were below the lowest limit of detection, while reaching values of only −0.5 to −2 $\log_{10}$ cfu only for intracellular bacteria.

The combined results for all strains and isolates shown in Figure 1 are shown graphically in Figure 2 as a function of multiples of their MIC, with the corresponding pertinent pharmacodynamic parameters presented in Table 3. It clearly appears that: (i) all strains showed similar concentration-dependent curves when using equipotent concentrations of ceftazidime; (ii) an intense bactericidal effect (E_{max} values close to the limit of detection) was globally obtained for the extracellular forms; (iii) conversely, the intracellular forms of all strains tested showed only a weak cfu decrease compared with the original, post-phagocytosis inoculum (E_{max} at around −1 $\log_{10}$ cfu); and (iv) that for both extracellular and intracellular forms, C_s values were close to the MICs.

We expanded the study by examining additional clinical isolates from group 1 [PA128 and PA129 (susceptible to ceftazidime in the absence of avibactam and with no or only a minor decrease in MIC by addition of avibactam)], group 2 [PA27, PA65, PA139, PA156 and PA281 (full restoration of ceftazidime activity at a 4 mg/L avibactam concentration)] and group 3 [PA254 and PA258 (no restoration of ceftazidime by avibactam)]. The results essentially confirmed the data obtained so far, namely that: (i) intracellular E_{max} values of ceftazidime for all isolates were limited to a maximum of −1.6 $\log_{10}$ cfu (except for PA254 for which E_{max} was −3.4 and −2.1 $\log_{10}$ cfu when exposed to ceftazidime alone or to ceftazidime combined with avibactam, respectively); and (ii) C_s values of ceftazidime against isolates for which avibactam reduced the MIC in broth were also shifted in a commensurate manner towards lower values upon addition of avibactam, while no meaningful change was seen for the isolates against which no effect of avibactam was seen in broth.

The impact of the addition of avibactam on the simultaneous decrease in the MIC and in the intracellular C_s is shown graphically in Figure 3 for five laboratory strains and eight clinical isolates for which detailed data were available. In the absence of avibactam, most strains (except PA156 and PA139) were either with both an MIC and an intracellular $C_s \leq 8$ mg/L ($n = 5$) or with both an MIC and a C_s above these values ($n = 6$). In the presence of avibactam, all but one strain (M1405 CON) had both an MIC and a $C_s \leq 8$ mg/L, indicating that avibactam restored the relative potency of ceftazidime to a similar extent whether acting on extracellular (broth) or intracellular *P. aeruginosa*.

Cellular penetration of avibactam

The penetration of avibactam in cells was assessed by comparing its total cellular concentration with its extracellular concentration in THP-1 monocytes after incubation with avibactam at two extracellular concentrations, in the presence of increasing concentrations of ceftazidime, and over time. Figure 4 shows that the concentration of avibactam in cells was consistently close to its extracellular concentration, without any statistically significant influence being exerted by its own concentration (4 or 10 mg/L) or by the presence of ceftazidime (0–160 mg/L), and with an apparent equilibrium achieved within 2 h.

Discussion

The present study extends our knowledge of the properties of avibactam by showing restoration of the activity of ceftazidime against β-lactamase-producing *P. aeruginosa* when bacteria are phagocytosed and thrive intracellularly. We used a validated pharmacodynamic model already applied to the study of a large number of approved and experimental antibiotics when acting against

Table 3. Pertinent regression parameters and goodness of fit of the 24 h dose–response curves of ceftazidime alone (CAZ) and of ceftazidime combined with avibactam at a fixed concentration (4 mg/L; CAZ/AVI) for extracellular (broth) and intracellular (THP-1 cells) activity of ceftazidime when pooling data from all strains presented individually in Figure 2 (ATCC 27853, PA152, PA315, M1405 def, M1405 CON, 2297 def and 2297 CON) as shown collectively, and normalized by MIC, in Figure 3

Activity	Condition	E_{min}[a] ($\Delta \log_{10}$ cfu) (CI)	E_{max}[b] ($\Delta \log_{10}$ cfu) (CI)	EC_{50}[c] ($\Delta \log_{10}$ cfu) (CI)	C_s[d] (× MIC)	R^2[e]
Extracellular	CAZ	3.9 (3.6 to 4.2)	−5.3 (−5.8 to − 4.7)	1.1 (0.9 to 1.4)	0.5	0.83
	CAZ/AVI	3.8 (3.5 to 4.2)	−5.47 (−5.8 to − 5.1)	1.2 (1.0 to 1.5)	0.5	0.84
Intracellular	CAZ	3.1 (2.8 to 3.4)	−1.1 (−1.3 to − 0.8)	0.2 (0.1 to 0.2)	0.8	0.90
	CAZ/AVI	3.0 (2.7 to 3.4)	−1.1 (−1.3 to − 0.9)	0.2 (0.1 to 0.3)	0.8	0.93

Parameters were calculated by fitting a single Hill–Langmuir function (sigmoidal equation with slope factor = 1) to each of the four datasets (ceftazidime intracellular/extracellular; ceftazidime/avibactam intracellular/extracellular) and are shown with their 95% CI where applicable.
[a]cfu increase (in $\log_{10}$ units) at 24 h from the corresponding initial inoculum as extrapolated from infinitely low antibiotic concentration using the Hill–Langmuir equation [= minimal pharmacological effect (minimal relative antibacterial efficacy in the model), corresponding to bacterial growth in the absence of antibiotic].
[b]cfu decrease (in $\log_{10}$ units) at 24 h from the corresponding initial inoculum as extrapolated from infinitely large antibiotic concentration using the Hill–Langmuir equation [= maximal pharmacological effect (maximal relative antibacterial efficacy in the model), corresponding to the maximal bacterial eradication that can be obtained with the antibiotic]; the practical limit of detection in our experiments was -5 $\log_{10}$ cfu from the initial inoculum (time 0 h).
[c]Extracellular concentration (in multiples of the MIC; total drug) at which the change in cfu was halfway between E_{min} and E_{max} using the Hill–Langmuir equation (this often-used parameter in drug pharmacodynamic analyses is useful for confirming the similarities of responses between two conditions, such as in this case absence or presence of avibactam, but does not have a direct microbiological significance).
[d]Extracellular concentration (in multiples of the MIC; total drug) at which there was no apparent change in cfu compared with the original inoculum, as determined by graphical interpolation using the Hill–Langmuir equation.
[e]Goodness of fit.

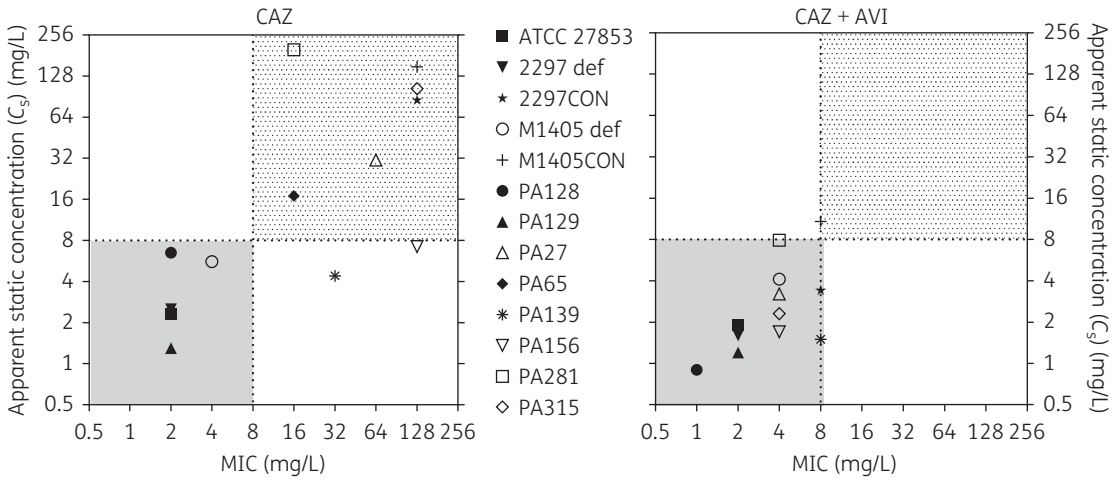

Figure 3. Correlation between the MIC of ceftazidime in broth (abscissa) for five laboratory strains (ATCC 27853, 2297 def, 2297CON, M1405 def and M1405CON) and eight clinical isolates (PA, see Table 1) and the corresponding apparent static concentrations (C_s) of ceftazidime (ordinate) against their intracellular forms [MIC data are from Table 1 and C_s values are from Table 2 or from additional identical experiments with the other isolates (curves not shown)]. Left panel: ceftazidime alone (CAZ). Right panel: ceftazidime combined with 4 mg/L avibactam (CAZ + AVI). The vertical broken line shows the EUCAST breakpoint for ceftazidime (resistance is greater than this limit) and the horizontal broken line shows the same value for C_s.

intracellular bacteria.[2] The data may have both pharmacological and clinical significance.

Examining the data in a pharmacological context, we see first that addition of avibactam to ceftazidime in the extracellular medium resulted in phagocytosed bacteria behaving essentially like fully susceptible ones with respect to the extracellular concentration needed to obtain a static effect (C_s, relative potency). Also, comparing ceftazidime potencies between extracellular and intracellular bacteria on the one hand, and ceftazidime/avibactam potencies likewise on the other, leads us to suggest that avibactam is able to freely enter THP-1 monocytes and reach β-lactamases present in the intermembrane space of phagocytosed bacteria in an unhindered fashion compared with bacteria in broth. Avibactam is expected to be negatively charged at pH 5–8 (based on Reaxys® version 2.20770.1, www.reaxys.com), which should prevent it from accumulating in cells.[26] Yet, the direct measurement of its penetration into THP-1 monocytes shows an apparent total concentration reflecting almost exactly its extracellular one. The data were also

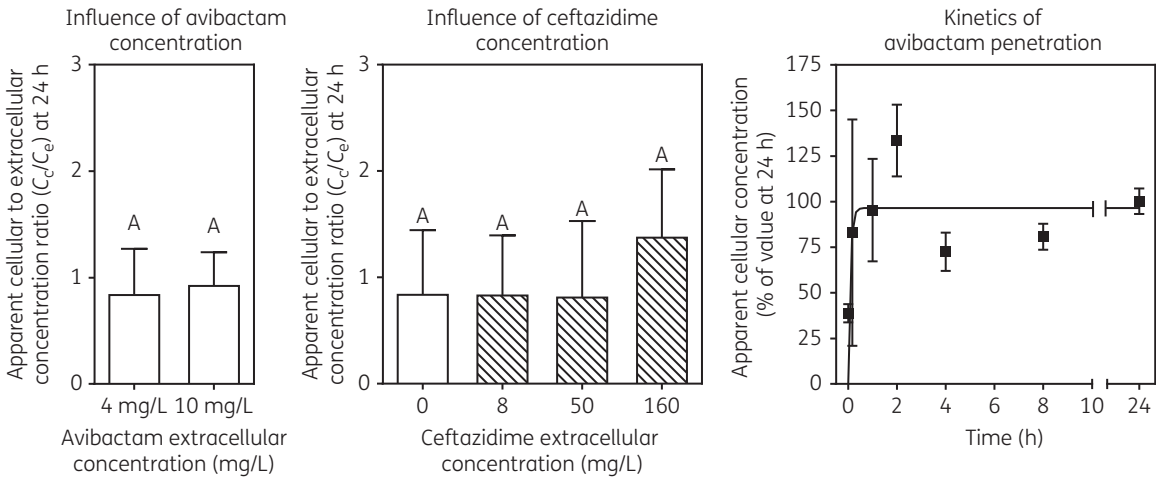

Figure 4. Cellular penetration of avibactam in THP-1 monocytes. Left panel: apparent cellular to extracellular concentration ratio after 24 h of incubation at two extracellular concentrations of avibactam. Middle panel: apparent cellular to extracellular concentration ratio after 24 h of incubation with 4 mg/L avibactam alone or together with increasing concentrations of ceftazidime. Right panel: apparent cellular concentration after incubation with avibactam at 4 mg/L for increasing time periods. Statistical analysis (left and middle panels): bars with the same letter are not significantly different from each other (unpaired two-tailed *t*-test).

consistent with ceftazidime being able to reach its intracellular target as it does it for bacteria in broth. This was actually already observed for other β-lactams when tested against phagocytosed *P. aeruginosa*,[16] and was also observed for many other antibiotics for the same bacteria[16] as well as for *S. aureus*[17,25,27,28] (see also Buyck *et al.*[2]). A marked exception, however, was seen for aminoglycosides for which intracellular potencies were lower than in broth (C_s values higher than MICs), due probably to the defeating effect exerted by the low pH prevailing in the phagolysosomes on the activity of these antibiotics (see discussion in Tulkens and Trouet[23]) and demonstrating the ability of the model to apprehend such differences.

A second pharmacological observation is that the intracellular maximal relative effect of ceftazidime (E_{max}, maximal relative efficacy in the model), even in the presence of avibactam, was only a minor fraction of what can be observed in broth. Once again, this has been observed quite systematically when assessing the activity of many different antibiotics, and especially β-lactams.[16,25] It is important to emphasize that E_{max} values are extrapolated values for an infinitely large extracellular antibiotic concentration, corresponding to the maximal effect (and, therefore maximal efficacy in the model) that can be obtained with the antibiotic even when pushed beyond the highest tested concentration (assuming that the function describing the antibiotic concentration–effect relationship remains the same as the one fitted to the actual data). Thus, ceftazidime (with or without avibactam), as many other antibiotics (see Van Bambeke and Tulkens[29] for a list of examples), appears unable to eradicate phagocytosed bacteria not through lack of potency (as discussed above) but because of lack of efficacy. Thus, part of the intracellular inoculum may not respond to the presence of the antibiotic (discussed in Buyck *et al.*,[2] Van Bambeke and Tulkens[29] and Van Bambeke *et al.*[30]), which may explain the relapsing and recurrent character of the infections where the intracellular inoculum represents an important part of the bacterial load (see examples in Shigeoka and Hill[31] and Bayston *et al.*[32] and in Drilling *et al.*[33] and Hamza *et al.*[34] for *P. aeruginosa* and *S. aureus*, respectively).

Moving now to the clinical significance of our data, they clearly indicate that intracellular inocula of bacteria producing β-lactamases are no more protected from avibactam than bacteria in broth. This is reassuring as the main indications for which ceftazidime/avibactam is approved may entail substantial intracellular inocula, due to phagocytosis of the offending organisms in lung and peritoneal macrophages[35–37] as well as urinary tract cells.[38] This supports using avibactam to restore ceftazidime activity in these approved indications. More broadly speaking, our studies call for similar investigations with already approved as well as with other novel β-lactamase inhibitors to see how they compare with avibactam for restoring susceptibility to intracellular forms of *P. aeruginosa* and other Gram-negative bacteria when these produce β-lactamase(s).

The present study has several limitations. First, we do not know what the subcellular distribution of avibactam is and have not directly assessed its assumed binding to and inactivation *in situ* of the β-lactamases produced by *P. aeruginosa* in phagocytes. This would require detailed drug disposition and metabolic studies in both non-infected and infected cells. The model also explores only one time point (24 h) due to intrinsic limitations (lack of growth before 8–10 h; explosive growth after 30 h in the absence of antibiotics) discussed previously.[2,16] The model is also a pharmacological one assessing the intracellular activity of antibiotics but not the cooperation between host cells and antibiotics, as unstimulated THP-1 monocytes show rather weak defences against invading bacteria (see discussion in Carryn *et al.*[39]). Next, the intracellular concentrations of ceftazidime were not measured, but we know that β-lactams, generally speaking, do not accumulate in eukaryotic cells and rather tend to reach cell concentrations similar to the extracellular ones.[23,30] Our data also show that a fixed concentration of avibactam of 4 mg/L may be insufficient to completely inhibit the activity of β-lactamase(s) of some *P. aeruginosa* clinical isolates encountered in the hospital from which they were collected. This was also observed among a small number of ceftazidime-resistant isolates of *P. aeruginosa* selected by others for *in vivo*

pharmacodynamics studies.[40] Our study, however, was neither designed nor powered as a true epidemiological survey since the isolates were selected for study based on their retrospectively known special phenotypic properties. The data must therefore be considered only as indicative. We note that similar *in vitro* observations have also been made for the combination of avibactam with aztreonam,[41] suggesting that detailed efficacy studies may be of interest. Lastly, we only examined one bacterial species, one β-lactam and one β-lactamase inhibitor, which means that the results cannot be extrapolated to other Gram-negative β-lactamase-producing bacteria or to other β-lactam-β-lactamase inhibitor combinations. This could be addressed in the future using the tools reported here.

Acknowledgements

We are grateful to Professor D. Livermore (University of East Anglia, Norwich, UK) for the kind gift of engineered strains, and to Dr H. Rodriguez-Villalobos (Cliniques universitaires St-Luc, Brussels, Belgium) for genotyping detection of β-lactamases in the clinical isolates used in this study. Ms M. C. Cambier and K. Santos Saial provided expert technical assistance. We thank all the manufacturers for the kind gift of the corresponding antibiotics.

Funding

This work was supported by a grant-in-aid from AstraZeneca Pharmaceuticals, Waltham, MA, USA and Forest/Cerexa, Oakland, CA, USA and by the Belgian Fonds de la Recherche Scientifique (grant numbers 2.4555.08, 1.5.034.10 and 3.4530.12). J. M. B. was supported by the Belgian Région Wallonne through the programme 'Aides à la Recherche et à l'Innovation technologique'.

Transparency declarations

J. M. B. was a postdoctoral fellow from the Region Wallonne. G. G. M. is an employee of the Université catholique de Louvain. F. V. B. is Senior Research Associate of the Belgian Fonds de la Recherche Scientifique (FRS-FNRS). K. M. K. and W. W. N. were employees of Cerexa Inc. and AstraZeneca Pharmaceuticals, respectively. C. L. was a student and P. M. T. an emeritus professor, both being unpaid and having nothing to declare.

Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online (https://academic.oup.com/jac).

References

1 Ruppé E, Woerther PL, Barbier F. Mechanisms of antimicrobial resistance in Gram-negative bacilli. *Ann Intensive Care* 2015; **5**: 61.

2 Buyck JM, Lemaire S, Seral C *et al*. In vitro models for the study of the intracellular activity of antibiotics. *Methods Mol Biol* 2016; **1333**: 147–57.

3 Bonnefoy A, Dupuis-Hamelin C, Steier V *et al*. In vitro activity of AVE1330A, an innovative broad-spectrum non-β-lactam β-lactamase inhibitor. *J Antimicrob Chemother* 2004; **54**: 410–7.

4 Robbins MJ, Cassettari M, Dencer C *et al*. In vitro activity of NXL104 (NXL), a new β-lactamase inhibitor, in combination with cefpodoxime (CPD) and cefixime (CFM) against 3rd generation cephalosporin-resistant isolates of species of the Enterobacteriaceae. In: *Abstracts of the Forty-Fifth Interscience Conference on Antimicrobial Agents and Chemotherapy*, Washington, DC, 2005. Abstract F-1161, p. 187. American Society for Microbiology, Washington, DC, USA.

5 Livermore DM, Mushtaq S, Warner M *et al*. NXL104 combinations versus Enterobacteriaceae with CTX-M extended-spectrum β-lactamases and carbapenemases. *J Antimicrob Chemother* 2008; **62**: 1053–6.

6 Wang DY, Abboud MI, Markoulides MS *et al*. The road to avibactam: the first clinically useful non-β-lactam working somewhat like a β-lactam. *Future Med Chem* 2016; **8**: 1063–84.

7 Bush K. A resurgence of β-lactamase inhibitor combinations effective against multidrug-resistant Gram-negative pathogens. *Int J Antimicrob Agents* 2015; **46**: 483–93.

8 Zhanel GG, Lawson CD, Adam H *et al*. Ceftazidime-avibactam: a novel cephalosporin/β-lactamase inhibitor combination. *Drugs* 2013; **73**: 159–77.

9 Mushtaq S, Warner M, Livermore DM. In vitro activity of ceftazidime+NXL104 against *Pseudomonas aeruginosa* and other non-fermenters. *J Antimicrob Chemother* 2010; **65**: 2376–81.

10 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.

11 Flamm RK, Stone GG, Sader HS *et al*. Avibactam reverts the ceftazidime MIC_{90} of European Gram-negative bacterial clinical isolates to the epidemiological cut-off value. *J Chemother* 2014; **26**: 333–8.

12 Sader HS, Castanheira M, Flamm RK *et al*. Antimicrobial activity of ceftazidime-avibactam against Gram-negative organisms collected from U.S. medical centers in 2012. *Antimicrob Agents Chemother* 2014; **58**: 1684–92.

13 Nichols WW, de Jonge BLM, Kazmierczak KM *et al*. In vitro susceptibility of global surveillance isolates of *Pseudomonas aeruginosa* to ceftazidime-avibactam (INFORM 2012 to 2014). *Antimicrob Agents Chemother* 2016; **60**: 4743–9.

14 US FDA, Silver Spring, MD, USA. *Avycaz Prescribing Information*. http://www.accessdata.fda.gov/drugsatfda_docs/label/2015/206494lbl.pdf.

15 EMA, London, UK. *Zavicefta Summary of Product Characteristics*. http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Product_Information/human/004027/WC500210234.pdf.

16 Buyck JM, Tulkens PM, Van Bambeke F. Pharmacodynamic evaluation of the intracellular activity of antibiotics towards *Pseudomonas aeruginosa* PAO1 in a model of THP-1 human monocytes. *Antimicrob Agents Chemother* 2013; **57**: 2310–8.

17 Barcia-Macay M, Lemaire S, Mingeot-Leclercq MP *et al*. Evaluation of the extracellular and intracellular activities (human THP-1 macrophages) of telavancin versus vancomycin against methicillin-susceptible, methicillin-resistant, vancomycin-intermediate and vancomycin-resistant *Staphylococcus aureus*. *J Antimicrob Chemother* 2006; **58**: 1177–84.

18 Livermore DM, Yang YJ. β-Lactamase lability and inducer power of newer β-lactam antibiotics in relation to their activity against β-lactamase-inducibility mutants of *Pseudomonas aeruginosa*. *J Infect Dis* 1987; **155**: 775–82.

19 Livermore DM. Interplay of impermeability and chromosomal β-lactamase activity in imipenem-resistant *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 1992; **36**: 2046–8.

20 Riou M, Carbonnelle S, Avrain L *et al*. In vivo development of antimicrobial resistance in *Pseudomonas aeruginosa* strains isolated from the lower respiratory tract of intensive care unit patients with nosocomial pneumonia and receiving antipseudomonal therapy. *Int J Antimicrob Agents* 2010; **36**: 513–22.

21 Clinical and Laboratory Standard Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Twenty-Fourth Informational Supplement M100-S24*. CLSI, Wayne, PA, USA, 2014.

22 Bogaerts P, Rezende de Castro R, de Mendonca R *et al*. Validation of carbapenemase and extended-spectrum β-lactamase multiplex endpoint PCR assays according to ISO 15189. *J Antimicrob Chemother* 2013; **68**: 1576–82.

23 Tulkens P, Trouet A. The uptake and intracellular accumulation of aminoglycoside antibiotics in lysosomes of cultured rat fibroblasts. *Biochem Pharmacol* 1978; **27**: 415–24.

24 Steinman RM, Brodie SE, Cohn ZA. Membrane flow during pinocytosis. A stereologic analysis. *J Cell Biol* 1976; **68**: 665–87.

25 Melard A, Garcia LG, Das D *et al*. Activity of ceftaroline against extracellular (broth) and intracellular (THP-1 monocytes) forms of methicillin-resistant *Staphylococcus aureus*: comparison with vancomycin, linezolid and daptomycin. *J Antimicrob Chemother* 2013; **68**: 648–58.

26 de Duve C, de Barsy T, Poole B *et al*. Commentary. Lysosomotropic agents. *Biochem Pharmacol* 1974; **23**: 2495–531.

27 Lemaire S, Glupczynski Y, Duval V *et al*. Activities of ceftobiprole and other cephalosporins against extracellular and intracellular (THP-1 macrophages and keratinocytes) forms of methicillin-susceptible and methicillin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2009; **53**: 2289–97. PM:19289525.

28 Peyrusson F, Butler D, Tulkens PM *et al*. Cellular pharmacokinetics and intracellular activity of the novel peptide deformylase inhibitor GSK1322322 against *Staphylococcus aureus* laboratory and clinical strains with various resistance phenotypes: studies with human THP-1 monocytes and J774 murine macrophages. *Antimicrob Agents Chemother* 2015; **59**: 5747–60.

29 Van Bambeke F, Tulkens PM, Limited maximal activity without marked loss of potency of antibiotics against intracellular forms of *Staphylococcus aureus* and *Pseudomonas aeruginosa*: an analysis with 13 bactericidal antibiotics from 7 different pharmacological classes in a pharmacodynamic model of human THP-1 infected monocytes. Poster Saturday-571. In: *Abstracts of ASM Microbe 2016*, Boston, MA, USA, 2016. American Society for Microbiology, Washington DC, USA.

30 Van Bambeke F, Barcia-Macay M, Lemaire S *et al*. Cellular pharmacodynamics and pharmacokinetics of antibiotics: current views and perspectives. *Curr Opin Drug Discov Dev* 2006; **9**: 218–30.

31 Shigeoka AO, Hill HR. Recurrent *Pseudomonas* infection associated with neutrophil dysfunction. *Scand J Infect Dis* 1978; **10**: 307–11.

32 Bayston R, Andrews M, Rigg K *et al*. Recurrent infection and catheter loss in patients on continuous ambulatory peritoneal dialysis. *Perit Dial Int* 1999; **19**: 550–5.

33 Drilling A, Coombs GW, Tan H *et al*. Cousins, siblings, or copies: the genomics of recurrent *Staphylococcus aureus* infections in chronic rhinosinusitis. *Int Forum Allergy Rhinol* 2014; **4**: 953–60.

34 Hamza T, Dietz M, Pham D *et al*. Intra-cellular *Staphylococcus aureus* alone causes infection in vivo. *Eur Cell Mater* 2013; **25**: 341–50.

35 Schmiedl A, Kerber-Momot T, Munder A *et al*. Bacterial distribution in lung parenchyma early after pulmonary infection with *Pseudomonas aeruginosa*. *Cell Tissue Res* 2010; **342**: 67–73.

36 Takajo D, Iwaya K, Katsurada Y *et al*. Community-acquired lobar pneumonia caused by *Pseudomonas aeruginosa* infection in Japan: a case report with histological and immunohistochemical examination. *Pathol Int* 2014; **64**: 224–30.

37 McClure CD, Schiller NL. Inhibition of macrophage phagocytosis by *Pseudomonas aeruginosa* rhamnolipids in vitro and in vivo. *Curr Microbiol* 1996; **33**: 109–17.

38 Nakao M, Kondo M, Imada A *et al*. An electron microscopic study of pathogenesis of urinary tract infection caused by *Pseudomonas aeruginosa* P 9 in mice. *Zentralbl Bakteriol Mikrobiol Hyg A* 1985; **260**: 369–78.

39 Carryn S, Van de Velde S, Van Bambeke F *et al*. Impairment of growth of *Listeria monocytogenes* in THP-1 macrophages by granulocyte macrophage colony-stimulating factor: release of tumor necrosis factor-α and nitric oxide. *J Infect Dis* 2004; **189**: 2101–9.

40 Berkhout J, Melchers MJ, van Mil AC *et al*. Pharmacodynamics of ceftazidime and avibactam in neutropenic mice with thigh or lung infection. *Antimicrob Agents Chemother* 2016; **60**: 368–75.

41 Sy SKB, Beaudoin ME, Zhuang L *et al*. In vitro pharmacokinetics/pharmacodynamics of the combination of avibactam and aztreonam against MDR organisms. *J Antimicrob Chemother* 2016; **71**: 1866–80.

Supplementary data

Figure S1. MICs of ceftazidime in the presence of increasing concentration of avibactam against *P. aeruginosa* strains: **A**, strains susceptible to ceftazidime, **B**, strains with resistance to ceftazidime counteracted with 4 mg/L of avibactam; **C**, strains with resistance to ceftazidime counteracted with >4 mg/L of avibactam; **D**, strains with resistance to ceftazidime not counteracted by avibactam