Efficacy of a Ceftazidime-Avibactam Combination in a Murine Model of Septicemia Caused by *Enterobacteriaceae* Species Producing AmpC or Extended-Spectrum β -Lactamases

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Avibactam is a novel non- β -lactam β -lactamase inhibitor that has been shown *in vitro* to inhibit class A, class C, and some class D β -lactamases. It is currently in phase 3 of clinical development in combination with ceftazidime. In this study, the efficacy of ceftazidime-avibactam was evaluated in a murine septicemia model against five ceftazidime-susceptible (MICs of 0.06 to 0.25 μ g/ml) and 15 ceftazidime-resistant (MICs of 64 to >128 μ g/ml) species of *Enterobacteriaceae*, bearing either TEM, SHV, CTX-M extended-spectrum, or AmpC β -lactamases. In the first part of the study, ceftazidime-avibactam was administered at ratios of 4:1 and 8:1 (wt/wt) to evaluate the optimal ratio for efficacy. Against ceftazidime-susceptible isolates of *Klebsiella pneumoniae* and *Escherichia coli*, ceftazidime and ceftazidime-avibactam demonstrated similar efficacies (50% effective doses [ED₅₀] of <1.5 to 9 mg/kg of body weight), whereas against ceftazidime-resistant β -lactamase-producing strains (ceftazidime ED₅₀ of >90 mg/kg), the addition of avibactam restored efficacy to ceftazidime (ED₅₀ dropped to <5 to 65 mg/kg). In a subsequent study, eight isolates (two AmpC and six CTX-M producers) were studied in the septicemia model. Ceftazidime-avibactam was administered at a 4:1 (wt/wt) ratio, and the efficacy was compared to that of the 4:1 (wt/wt) ratio of either piperacillin-tazobactam or cefotaxime-avibactam. Against the eight isolates, ceftazidime-avibactam was the more effective combination, with ED₅₀ values ranging from 2 to 27 mg/kg compared to >90 mg/kg and 14 to >90 mg/kg for piperacillin-tazobactam and cefotaxime-avibactam, respectively. This study demonstrates that the potent *in vitro* activity observed with the ceftazidime-avibactam combination against ceftazidime-resistant *Enterobacteriaceae* species bearing class A and class C β -lactamases translated into good efficacy in the mouse septicemia model.

Bacterial resistance plays a prominent role in determining treatment options and currently represents a major public health issue. The β -lactam antibiotics are active against a wide range of bacterial pathogens and have low toxicity to humans, so the globally increasing levels of resistance to these agents are a particularly serious concern (1, 2). In Gram-negative organisms, one of the most important mechanisms of resistance to β -lactams is the enzymatic cleavage of the β -lactam ring by β -lactamases (3). These enzymes are grouped into four classes based on their amino acid sequences. The class A, C, and D β -lactamases contain a serine residue at the catalytic site, while the class B enzymes contain one or more zinc atoms (4). One very successful strategy to overcome β -lactamase-mediated resistance is to combine the β -lactam antibiotic with a β -lactamase inhibitor, such as clavulanic acid, tazobactam, or sulbactam. These currently marketed β -lactamase inhibitors have a limited spectrum of clinical utility as their inhibitory activity is confined, generally, to the class A and a few class D β -lactamases.

Avibactam is the first of a new class of non- β -lactam β -lactamase inhibitors referred to as diazabicyclooctanes (5). It displays a broad spectrum of inhibitory activity against both the class A and class C β -lactamases, inactivating the enzymes efficiently at low 50% inhibitory concentrations [IC₅₀], with low turnover numbers (6, 7). It has very little intrinsic antibacterial activity but efficiently protects β -lactams from hydrolysis by a wide variety of class A-, class C-, and some class D-producing strains (8–13), including extended-spectrum β -lactamases (ESBLs) (14), *Klebsiella pneumoniae* carbapenemase (KPC) (15, 16), and OXA-48 producers (17). The β -lactamase landscape is changing radically, with KPCs and CTX-M-type ESBLs now causing major resistance problems

around the world (18, 19). Both *in vitro* and *in vivo* studies of combinations of oxymino-cephalosporins with avibactam have been reported to overcome these resistances (20–22). While there is an extensive body of literature on the *in vitro* activities of ceftazidime-avibactam combinations, only a few studies on the *in vivo* efficacy of this combination against *Escherichia coli*, *K. pneumoniae*, *Enterobacter cloacae*, *Citrobacter freundii*, and *Pseudomonas aeruginosa* have been published (20–27).

In this study, 20 isolates of *Enterobacteriaceae* were studied in a murine acute lethal septicemia model, with most isolates producing ESBLs and/or AmpC β -lactamases. The objectives of the study were to (i) evaluate the *in vivo* efficacy of ceftazidime with or without avibactam at two different ratios (4:1 and 8:1) and compare the relative efficacy of the combination to that of commer-

Received 13 June 2014 Returned for modification 8 July 2014

Accepted 10 August 2014

Published ahead of print 18 August 2014

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.03579-14>.

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doi:10.1128/AAC.03579-14

TABLE 1 MICs against isolates of *Enterobacteriaceae* reported in Table 3

			MIC (μg/ml) for:					
			Ceftazidime					
			+ Avibactam					
Isolate	Phenotype	β-Lactamase	Alone	4 μg/ml ^a	4:1 ^b	8:1 ^c	Amoxicillin-clavulanate ^d	Piperacillin-tazobactam ^e
<i>E. coli</i> 250GR12	CAZ-S		0.06	0.06	0.06	0.06	2	2
<i>E. coli</i> 250GR43	CAZ-S		0.25	0.12	0.12	0.12	4	1
<i>K. pneumoniae</i> 283GR4	CAZ-S		0.25	0.5	0.25	0.25	16	32
<i>K. pneumoniae</i> 283IP53	CAZ-S		≤0.12	≤0.12	0.12	0.12	4	4
<i>P. stuartii</i> 321UC1	CAZ-S		1	0.5	0.5	1	64	4
<i>E. coli</i> 250BE1	CAZ-R	SHV-4	≥128	0.25	2	4	8	16
<i>K. pneumoniae</i> 283IP10	CAZ-R	AmpC; SHV-4	64	0.5	1	2	8	>32
<i>K. pneumoniae</i> 283IP35	CAZ-R	SHV-2	≥128	0.25	0.5	1	64	32
<i>K. pneumoniae</i> 283IP84	CAZ-R	TEM-3	64	0.25	2	8	32	32
<i>E. cloacae</i> 293GR8	CAZ-R	AmpC	≥128	0.5	1	2	≥128	32
<i>E. cloacae</i> 293GR38	CAZ-R	AmpC	64	0.5	1	1	64	>32
<i>E. cloacae</i> P99	CAZ-R	AmpC	≥128	0.5	0.5	1	≥128	>32
<i>C. freundii</i> 261GR3	CAZ-R	AmpC	≥128	0.06	1	1	≥128	32
<i>C. freundii</i> 261GR6	CAZ-R	AmpC	≥128	0.5	2	2	≥128	32

^a MICs for ceftazidime in the presence of a fixed 4- μ g/ml avibactam concentration.^b MICs for ceftazidime when combined with avibactam in a 4:1 (wt/wt) ratio.^c MICs for ceftazidime when combined with avibactam in a 8:1 (wt/wt) ratio.^d Commercially available amoxicillin-clavulanate (1/0.2 g) (Augmentin).^e Commercially available piperacillin-tazobactam (4/0.5 g) (Tazocillin).

cially available amoxicillin-clavulanate (2:1 ratio) and piperacillin-tazobactam (8:1 ratio) and (ii) evaluate the efficacy of ceftazidime, piperacillin, and cefotaxime with or without avibactam and/or tazobactam at a 4:1 ratio against AmpC- or CTX-M-producing *E. cloacae*, *E. coli*, and *K. pneumoniae* isolates. Some of this work was reported previously in abstract form (20, 21, 24).

MATERIALS AND METHODS

Antimicrobial test agents. Avibactam was supplied by Novexel (Romainville, France). Ceftazidime pentahydrate (Fortum) was supplied by Sandoz. Amoxicillin-clavulanate (1/0.2 g; Augmentin) was supplied by GlaxoSmithKline. Piperacillin-tazobactam (4/0.5 g; Tazocillin) was supplied by Wyeth-Lederle. Cefotaxime, piperacillin, and tazobactam were purchased from Sigma-Aldrich (France).

Bacterial isolates. Twenty different isolates of *Enterobacteriaceae*, 5 ceftazidime-susceptible and 15 ceftazidime-resistant isolates with different β -lactamases, were tested in this study. Seventeen clinical isolates were obtained from different French hospitals, including the CTX-M *E. coli* isolates, which were provided by Guillaume Arlet (Hôpital Tenon, Paris, France). Three CTX-M *K. pneumoniae* isolates were kindly provided by Robert Bonomo (Case Western Reserve University School of Medicine, Cleveland, OH, USA). The β -lactamases produced by these strains were well characterized and are listed in Tables 1 and 2.

Determination of β -lactamases. The determination and characterization of β -lactamases expressed in various bacterial strains were achieved by *bla* gene detection, eventually combined with isoelectric focusing (IEF) of cell extracts.

Gene detection. Bacterial DNA was subjected to PCR amplification profiling using the appropriate primers for detection of the most common *bla* gene families (28–30) (see the supplemental material): class A, TEM, SHV, VEB, PER, GES, CARB, CTX-M, and KPC families; class B, IMP and VIM families; class C, CMY-1/MOX, CMY-2, DHA, ACC, ACT-1, and FOX plasmidic subgroups; class D, OXA-1, OXA-2, OXA-10/13, and OXA-18/45 families.

The PCRs were performed using Ready-To-Go reagents (GE Healthcare), according to the manufacturer's instructions. Briefly, cell lysis was performed at 99°C for 3 min, followed by 30 cycles of amplification (94°C

for 1 min, 55°C for 1 min, and 72°C for 1 min) and a final extension at 72°C for 5 min.

In vitro susceptibility. MICs were determined using the guidelines of the Clinical and Laboratory Standards Institute (CLSI) for antimicrobial susceptibility testing with cation-adjusted Mueller-Hinton broth (31). The MIC was defined as the lowest concentration that inhibited visual growth. MICs for ceftazidime, piperacillin, or cefotaxime were determined with (i) avibactam or tazobactam at ratios of 4:1 and 8:1 and (ii) avibactam or tazobactam used at a fixed 4- μ g/ml concentration with various concentrations of either ceftazidime, piperacillin, or cefotaxime. The interpretive criteria considered for ceftazidime and cefotaxime in combination with 4 μ g/ml avibactam were those defined by the CLSI for each antibiotic alone (32). All other combinations employed a fixed ratio of the antibiotic to the inhibitor to help interpret the fixed ratio *in vivo* data rather than to determine sensitivity/resistance.

Mice. Male 5- to 6-week-old (20 to 23 g) ICR (CD-1) mice (Charles River Laboratories, France) were used in the acute lethal septicemia model. The mice were housed in groups of 5 to 10 with free access to food and water in the Microbiology In Vivo Laboratory (Antiinfective Research, Novexel, Romainville, France). Experiments were carried out according to the protocols approved by the institutional animal care and ethical committee (Novexel, Romainville, France) and authorization from the Département de Santé Vétérinaire, Prefecture de Bobigny, France.

Murine acute lethal septicemia. The mice were infected by intraperitoneal injection of the bacterial strains in 5% hog gastric mucin (Sigma) containing inocula of 1.5×10^8 to 2.7×10^9 CFU in a 0.5-ml volume. Groups of 10 mice were dosed subcutaneously with the antibiotic or the antibiotic-inhibitor combination at different doses (dose ranges of 1.5 mg/kg to 100 mg/kg of body weight) (1 dose per group) in 0.2 ml saline. Dosing was performed twice at 1 and 4 h postinfection. A control group of 10 to 15 infected mice received only saline at the dosing times.

In this model, the infected mice developed septicemia and became moribund within 48 h of infection unless they received adequate therapy. The efficacy was monitored using survival as the endpoint, with observation continued for 5 days posttreatment. The animals under test were inspected multiple times per day, and stressed animals were euthanized.

TABLE 2 MICs against isolates of *Enterobacteriaceae* reported in Table 4

Isolate	β -Lactamase	MIC (μ g/ml) for ^a :								
		Ceftazidime			Cefotaxime			Piperacillin		
		Alone	Plus avibactam		Alone	Plus avibactam		Alone	Plus tazobactam	
			4 μ g/ml	4:1		4 μ g/ml	4:1		4 μ g/ml	4:1
<i>K. pneumoniae</i> 283IP10	AmpC; SHV-4	≥ 128	0.5	1	≥ 128	0.25	NT ^b	≥ 128	64	≥ 128
<i>E. cloacae</i> P99	AmpC	≥ 128	0.5	0.5	≥ 128	0.25	NT	≥ 128	≥ 128	≥ 128
<i>E. coli</i> TN06	CTX-M-2; TEM-1	≥ 128	0.5	4	≥ 128	0.125	2	≥ 128	16	16
<i>E. coli</i> E4	CTX-M-16; TEM-1	≥ 128	1	4	≥ 128	0.5	1	≥ 128	8	8
<i>E. coli</i> TN03	CTX-M-15; TEM-1; OXA-1	≥ 128	0.25	2	≥ 128	≤ 0.125	0.5	≥ 128	2	2
<i>K. pneumoniae</i> 465	CTX-M-2; TEM-1B	64	2	4	≥ 128	0.25	4	≥ 128	≥ 128	≥ 128
<i>K. pneumoniae</i> 253	CTX-M-2; SHV-5; TEM-2	≥ 128	2	8	≥ 128	0.25	4	≥ 128	≥ 128	≥ 128
<i>K. pneumoniae</i> K4	CTX-M-15; TEM-1; OXA-1	≥ 128	1	4	≥ 128	≤ 0.125	4	≥ 128	≥ 128	≥ 128

^a MICs for ceftazidime, cefotaxime, and piperacillin were determined with avibactam or tazobactam at a ratio of 4:1 (wt/wt) and avibactam or tazobactam at a fixed 4 μ g/ml concentration.

^b NT, not tested.

The 50% effective dose (ED₅₀) is reported as the unit dose of the antibiotic component in mg/kg. As two doses for each dosage group (1 and 4 h postinfection) were utilized, the total ED₅₀ should be interpreted as the ED₅₀ $\times$ 2 mg/kg. For the antibiotic-inhibitor treatments, the dose reported is the unit dose of the antibiotic. Thus, a 4:1 ceftazidime-avibactam combination ED₅₀ of 10 mg/kg represents 10 mg/kg ceftazidime + 2.5 mg/kg avibactam. The ED₅₀ values were calculated by log-probit analysis (33) using software written in-house.

RESULTS AND DISCUSSION

In vitro susceptibility. Tables 1 and 2 show the MICs for ceftazidime, amoxicillin, cefotaxime, and piperacillin either alone or in combination with avibactam or tazobactam against ceftazidime-susceptible and -resistant isolates of *Enterobacteriaceae*. Using the CLSI-approved method of reporting the MICs of ceftazidime and cefotaxime, in the presence of a fixed 4 μ g/ml dose of avibactam, the activities of both ceftazidime and cefotaxime against the class A and class C β -lactamase-producing isolates were restored.

MICs were also determined using fixed antibiotic/inhibitor ratios, allowing a direct comparison with the *in vivo* data in Tables 3 and 4. Avibactam at both the 4:1 and 8:1 ratios significantly improved the *in vitro* activity of ceftazidime against both class A- and class C-producing isolates. Avibactam when combined with cefotaxime at a 4:1 ratio also significantly improved the activity of the antibiotic against AmpC- and CTX-M-producing *E. coli* and *K. pneumoniae* isolates, while piperacillin-tazobactam at a 4:1 ratio was not active against the AmpC-producing isolates (Table 2).

Saline-treated control animals. Mice infected with the ceftazidime-susceptible and -resistant *Enterobacteriaceae* isolates and treated with saline postinfection succumbed to the infection within 48 h (100% of animals), thereby demonstrating the pathogenicity of the isolates used in the study.

Studies with ceftazidime-avibactam at ratios of 4:1 and 8:1. In the initial studies, the optimal ratios of the antibiotic to the inhibitor for *in vivo* efficacy against the ceftazidime-susceptible

TABLE 3 Efficacy of ceftazidime-avibactam against ESBL- and AmpC-producing strains of *Enterobacteriaceae*

				ED ₅₀ (mg/kg [95% confidence limit]) for ^a :				
				Ceftazidime			Augmentin ^b	Tazocillin ^c
				Alone	Plus avibactam			
Isolate	Phenotype	β-Lactamase	MIC for ceftazidime (μg/ml)			4:1	8:1	
<i>E. coli</i> 250GR12	CAZ-S		0.06	<5	<5	<5	12 (5–25)	>50
<i>E. coli</i> 250GR43	CAZ-S		0.25	9 (3–17)	5 (1–8)	6 (4–11)	22 (14–36)	>50
<i>K. pneumoniae</i> 283GR4	CAZ-S		0.25	<1.5	<1.5	<1.5	>50	>50
<i>K. pneumoniae</i> 283IP53	CAZ-S		≤0.12	4 (0–5)	4.5 (0–5)	4.5 (0–5)	27 (0–107)	>50
<i>P. stuartii</i> 321UC1	CAZ-S		1	6 (1–14)	5 (1–10)	4 (1–7)	>50	>50
<i>E. coli</i> 250BE1	CAZ-R	SHV-4	≥128	>90	16 (10–27)	16 (10–27)	49 (28–533)	39 (10–192)
<i>K. pneumoniae</i> 283IP10	CAZ-R	AmpC; SHV-4	64	>90	5 (1–8)	9 (6–13)	20 (12–34)	>90
<i>K. pneumoniae</i> 283IP35	CAZ-R	SHV-2	>128	>90	29 (19–50)	18 (5–33)	>90	>90
<i>K. pneumoniae</i> 283IP84	CAZ-R	TEM-3	64	>90	<5	<5	43 (18–134)	>90
<i>E. cloacae</i> 293GR8	CAZ-R	AmpC	>128	>90	<10	11 (4–19)	>90	>90
<i>E. cloacae</i> 293GR38	CAZ-R	AmpC	64	>90	58 (38–93)	65 (41–126)	>90	>90
<i>E. cloacae</i> P99	CAZ-R	AmpC	>128	85 (49–75)	5 (3–9)	10 (0–10)	>90	43 (7–75)
<i>C. freundii</i> 261GR3	CAZ-R	AmpC	>128	>90	13 (11–15)	14 (11–26)	>90	>90
<i>C. freundii</i> 261GR6	CAZ-R	AmpC	>128	84 (61–145)	9 (0–10)	9 (0–10)	>90	>90

^a Mice were dosed twice at 1 and 4 h postinfection; the ED₅₀ of the antibiotic component is reported here.

^b Commercially available amoxicillin-clavulanate (1/0.2 g) (Augmentin).

^c Commercially available piperacillin-tazobactam (4/0.5 g) (Tazocillin).

Isolate	β -Lactamase	MIC for cefazidime ($\mu\text{g/ml}$)	ED ₅₀ (mg/kg [95% confidence limit]) for ^a :					
			Ceftazidime		Piperacillin		Cefotaxime	
			Alone	Plus avibactam, 4:1	Alone	Plus tazobactam, 4:1	Alone	Plus avibactam, 4:1
<i>K. pneumoniae</i> 283P10	AmpC; SHV-4	≥ 128	>90	7 (0–10)	>90	>90	NT ^b	NT
<i>E. cloacae</i> P99	AmpC	≥ 128	85 (49–75)	5 (3–9)	>90	>90	NT	NT
<i>E. coli</i> TN06	CTX-M-2; TEM-1	≥ 128	>90	21 (16–31)	>90	>90	>90	55 (44–70)
<i>E. coli</i> E4	CTX-M-16; TEM-1	≥ 128	74 (60–500)	13 (9–29)	>90	>90	>90	>90
<i>E. coli</i> TN03	CTX-M-15; TEM-1; OXA-1	≥ 128	>90	2 (1–4)	>90	>90	NT	NT
<i>K. pneumoniae</i> 465	CTX-M-2; TEM-1B	64	>90	18 (6–32)	>90	>90	>90	14 (6–61)
<i>K. pneumoniae</i> 253	CTX-M-2; SHV-5; TEM-2	≥ 128	127 (82–500)	27 (14–53)	>90	>90	165 (94–500)	70 (42–155)
<i>K. pneumoniae</i> K4	CTX-M-15; TEM-1; OXA-1	≥ 128	>90	21 (12–40)	>90	>90	NT	NT

^a Mice were dosed twice at 1 and 4 h postinfection; the ED₅₀ of the antibiotic component is reported here.

^b NT, not tested.

tors, including *in vitro*, *in vivo*, and the hollow-fiber infection model data (8, 9, 12, 13, 20, 21, 23–26, 34).

Eventually, eight isolates (two AmpC producers and six CTX-M producers) were studied at a 4:1 ratio of ceftazidime-avibactam, and the results were compared to those for piperacillin-tazobactam and cefotaxime-avibactam, respectively. The *in vivo* efficacies of ceftazidime-avibactam combinations were consistently better than that of other antibiotic- β -lactamase inhibitor combinations, such as piperacillin-tazobactam. Since tazobactam is a poor inhibitor of both these enzymes (35–37), it is not surprising that the piperacillin-tazobactam combination was uniformly less active, whereas the ceftazidime-avibactam combination afforded protection in the septicemia model. Furthermore, unlike clavulanic acid, avibactam did not induce the AmpC β -lactamase in three strains of *E. cloacae* (38).

Cefotaxime and cefotaxime-avibactam were used as comparators in studies on CTX-M-producing isolates. Although avibactam generally protected cefotaxime in these studies when dosed twice at 1 h and 4 h postinfection, protection was poor against some CTX-M producers, particularly *E. coli* E4. Dosing three times (at 1, 4, and 7 h postinfection) also failed to give a measurable ED₅₀ against this isolate, both cefotaxime and the cefotaxime-avibactam 4:1 ratio having ED₅₀ of >90 mg/kg (data not shown).

In conclusion, these data show that a 4:1 ratio of ceftazidime-avibactam proved effective against a range of *Enterobacteriaceae* isolates in a mouse septicemia model where ceftazidime alone was ineffective due to production of β -lactamases by the infecting organisms. Avibactam was also tested in combination with a number of other β -lactams, but none of these combinations had a potency equivalent to that of ceftazidime-avibactam. The KPC β -lactamases are an important cause of cephalosporin resistance, and no KPCs were included in this study. However, Endimiani et al. (22) have demonstrated *in vivo* the efficacy of ceftazidime-avibactam at a ratio of 4:1 against two *Enterobacteriaceae* strains producing KPC β -lactamases.

Ceftazidime-avibactam has successfully completed two phase 2 clinical studies (39, 40) and is currently in phase 3 clinical development for the treatment of complicated intra-abdominal infections, urinary tract infections, nosocomial pneumonia, and infections with ceftazidime-resistant pathogens (registration numbers NCT01499290, NCT01500239, NCT01726023, NCT01595438, NCT01599506, NCT01808092, and NCT01644663 at <http://clinicaltrials.gov>). Based on both preclinical and phase 2 data, ceftazidime-avibactam seems to be a promising treatment option against the widespread multidrug-resistant *Enterobacteriaceae* and *P. aeruginosa* isolates which currently pose a worldwide problem (41, 42).

ACKNOWLEDGMENTS

We thank R. Bonomo and G. Arlet for supplying some of the isolates used in this study.

This study was funded by Novexel.

Ceftazidime-avibactam is now being developed by AstraZeneca and Forest-Cerexa.

P.L., A.-M.G., C.M., and K.C. received compensation fees for services in relation to preparing the manuscript, which was funded by AstraZeneca. P.L., A.-M.G., L.L., C.M., J.P., and K.C. are former employees of Novexel.

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