AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

Impaired Inhibition by Avibactam and Resistance to the Ceftazidime-Avibactam Combination Due to the D^{179}Y Substitution in the KPC-2 β-Lactamase

Fabrice Compain,[a,b,c,d] **Michel Arthur**[a,b,c]

INSERM UMRS 1138,[a] Sorbonne Universités, UPMC Univ Paris 06,[b] Sorbonne Paris Cité, Université Paris Descartes, Université Paris Diderot,[c] Centre de Recherche des Cordeliers, Paris, France; Service de Microbiologie, Assistance Publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, Paris, France[d]

ABSTRACT The ceftazidime-avibactam antibiotic combination was recently shown to be at risk for the emergence of resistance under treatment. To gain insight into the underlying mechanism, we have analyzed the catalytic properties of a *Klebsiella pneumoniae* carbapenemase type 2 (KPC-2) β-lactamase harboring the D^{179}Y substitution. We show that impaired inhibition by avibactam combined with significant residual activity for ceftazidime hydrolysis accounts for the resistance. In contrast, the D^{179}Y substitution abolished the hydrolysis of aztreonam and imipenem, indicating that these drugs might provide therapeutic alternatives.

KEYWORDS β-lactamase inhibitor, avibactam, KPC-2, carbapenemase, ceftazidime

Received 2 March 2017 **Returned for modification** 19 April 2017 **Accepted** 26 April 2017

Accepted manuscript posted online 1 May 2017

Citation Compain F, Arthur M. 2017. Impaired inhibition by avibactam and resistance to the ceftazidime-avibactam combination due to the D^{179}Y substitution in the KPC-2 β-lactamase. Antimicrob Agents Chemother 61:e00451-17. https://doi.org/10.1128/AAC.00451-17.

Address correspondence to Michel Arthur, michel.arthur@crc.jussieu.fr.

Resistance to carbapenems in *Enterobacteriaceae* is often due to the production of class A β-lactamases belonging to the *Klebsiella pneumoniae* carbapenemase (KPC) type (1). The most commonly encountered variants worldwide are KPC-2 and KPC-3, which confer high-level resistance to most available β-lactams. The production of KPC β-lactamases is often associated with resistance to other classes of antibiotics, including aminoglycosides, fluoroquinolones, and colistin, leaving few or no therapeutic alternatives (2, 3). Inhibition of KPC enzymes by classical β-lactamase inhibitors (clavulanate, sulbactam, and tazobactam) is not sufficient to restore the activity of β-lactams (4). In this context, a new β-lactam–β-lactamase inhibitor combination, ceftazidime-avibactam (5), has recently obtained regulatory approval in the United States and Europe. The combination has a broad spectrum of activity against multidrug-resistant *Enterobacteriaceae* and *Pseudomonas aeruginosa* strains producing class A, C, and some class D β-lactamases (6, 7).

The emergence of resistance to the ceftazidime-avibactam combination has been recently reported in three out of 37 patients infected with carbapenem-resistant *Enterobacteriaceae* and treated with these drugs (8). The emergence of resistance, which was associated with microbiological failure, was due to amino acid substitutions in the KPC-3 enzymes produced by the *K. pneumoniae* isolates recovered from these three patients (D^{179}Y, V^{240}G, and D^{179}Y associated with T^{243}M) (9). The impact of these substitutions on the kinetic parameters for hydrolysis of ceftazidime and inhibition by avibactam has not been previously reported. In another study (10), site-directed mutagenesis identified rare substitutions resulting in resistance to the ceftazidime-avibactam combination. A comparison of KPC-2 and KPC-2 D^{179}N did not reveal any modification of the steady-state rate of ceftazidime hydrolysis or inhibition by avibactam (10). However, an initial burst of ceftazidime hydrolysis was detected for KPC-2 D^{179}N but not for the parental enzyme (10).

To gain insight into the modifications of the catalytic properties of KPC enzymes that

TABLE 1 Impact of D^{179}Y substitution on MICs of β-lactams against *E. coli* TOP10 producing a KPC-2 β-lactamase in the absence or presence of a β-lactamase inhibitor

	MIC (μg/ml) of indicated β-lactams with avibactam (+Avi), with clavulanate (+Clav), or in the absence of inhibitor (none)[a]									
	Ampicillin			Ceftazidime			Ceftriaxone	Aztreonam	Meropenem	Imipenem
β-Lactamase	None	+Avi	+Clav	None	+Avi	+Clav	None	None	None	None
None	2	2	2	0.25	0.25	0.25	<0.12	0.25	<0.12	0.25
KPC-2	>128	128	>128	>128	1	64	>128	>128	>128	128
KPC-2 D^{179}Y	16	8	8	>128	32	8	32	0.5	<0.12	0.25

[a]Inhibitors were used at a fixed concentration of 4 μg/ml. Data are the median of the results from five determinations.

lead to resistance to the ceftazidime-avibactam combination, we have isolated *in vitro* a mutant derived from a KPC-2-producing strain of *Escherichia coli*. The resulting KPC-2 enzyme, which was found to harbor the previously described D^{179}Y substitution, was kinetically characterized with respect to the hydrolysis of various β-lactams and the inhibition by avibactam.

***In vitro* selection for resistance to the ceftazidime-avibactam combination.** Selection was performed with *E. coli* TOP10 harboring plasmid pTRC-99kΩbla_{KPC-2}, which enables expression of the bla_{KPC-2} gene encoding KPC-2 under the control of the P_{trc} promoter (11). Approximately 6×10^9 CFU were plated on Mueller-Hinton (MH) agar (Difco) containing isopropyl-β-D-1-thiogalactopyranoside (IPTG) for the induction of P_{trc}, a fixed concentration of avibactam (4 μg/ml), and 2-fold-increasing concentrations of ceftazidime (1 to 32 μg/ml). CFU grew after 48 h of incubation at frequencies of 2×10^{-8}, 5×10^{-9}, and 1×10^{-9} on agar plates containing 4, 8, and 16 μg/ml ceftazidime, respectively. Sequencing of the bla_{KPC-2} gene from 10 putative mutants obtained at the highest concentrations of ceftazidime (8 or 16 μg/ml) revealed the same point mutation leading to the D^{179}Y substitution in 5 resistant clones and the wild-type sequence in the remaining clones. The plasmid encoding KPC-2 D^{179}Y was extracted and introduced by transformation into *E. coli* TOP10, with selection for resistance to kanamycin conveyed by the vector pTRC-99k. The resulting transformants grew in the presence of ceftazidime (8 μg/ml) and avibactam (4 μg/ml), indicating that the D^{179}Y substitution was sufficient for resistance to the drug combination, as these concentrations correspond to the susceptible breakpoints by FDA and EUCAST criteria.

Modification of the resistance phenotype resulting from the D^{179}Y substitution in KPC-2. The MICs of β-lactams were determined by the microdilution method in MH broth, according to Clinical and Laboratory Standards Institute (CLSI) recommendations (12). Avibactam and clavulanate were used at a fixed concentration of 4 μg/ml in combination with β-lactams. IPTG (500 μM) was added to the microdilution plates to induce production of the β-lactamase. The precultures were grown in MH broth containing IPTG (500 μM) and kanamycin (50 μg/ml) for plasmid maintenance.

E. coli TOP10 producing wild-type KPC-2 was resistant to all tested β-lactams (MIC, ≧128 μg/ml), whereas the ceftazidime-avibactam combination was active (MIC, 1 μg/ml), according to FDA and EUCAST interpretive criteria (Table 1). In contrast, avibactam did not restore susceptibility to ceftazidime in the strain producing the KPC-2 D^{179}Y variant (MIC, 32 μg/ml), as expected from the selection procedure. Resistance to ceftazidime alone was not abolished by D^{179}Y (MIC, >128 μg/ml), indicating that the β-lactamase remained active in the hydrolysis of this cephalosporin. The D^{179}Y substitution decreased the MICs of ampicillin, ceftriaxone, aztreonam, and meropenem, resulting in susceptibility for aztreonam and meropenem (MICs, 0.5 and <0.12 μg/ml, respectively). Interestingly, clavulanate significantly reduced the MIC of ceftazidime against *E. coli* TOP10 producing KPC-2 D^{179}Y (from >128 μg/ml to 8 μg/ml). In conclusion, the D^{179}Y substitution led to the acquisition of resistance to the ceftazidime-avibactam combination and of susceptibility to aztreonam and meropenem. These results are in full agreement with previous analyses of the consequences of the D^{179}Y substitution in various genetic backgrounds (9).

Impact of the D179Y substitution on the hydrolysis of β-lactams by KPC-2. Wild-type KPC-2 and KPC-2 D179Y were produced in *E. coli* TOP10 cells harboring pTRC-99kΩbla_{KPC-2} or its derivative encoding KPC-2 D179Y, as previously described (11). The β-lactamases were purified by anion-exchange and size-exclusion chromatography (11). All kinetic experiments were performed at 20°C in 2-(*N*-morpholino)ethanesulfonic acid (MES; 100 mM [pH 6.4]) in a Cary 300 spectrophotometer (Agilent), as previously described (11). Steady-state kinetic parameters (k_{cat}, K_m, and k_{cat}/K_m) were determined at a fixed concentration of the β-lactamase [E] and various concentrations of the substrate [S]. Initial rate (v_i) values were plotted as a function of [S], according to the equation $v_i = k_{cat}\,[E][S]/(K_m + [S])$. K_m values that could not be evaluated by this method were determined by analyzing competitive hydrolysis of the β-lactam and the chromogenic cephalosporin CENTA (13), as previously described (11, 14). Variations in the molar extinction coefficients ($\Delta\varepsilon$) resulting from hydrolysis of the β-lactam ring were 7,380 $M^{-1} \cdot cm^{-1}$ ($\lambda_{415\ nm}$) for CENTA, $-500\ M^{-1} \cdot cm^{-1}$ ($\lambda_{235\ nm}$) for ampicillin, $-9{,}800\ M^{-1} \cdot cm^{-1}$ ($\lambda_{256\ nm}$) for ceftazidime, $-400\ M^{-1} \cdot cm^{-1}$ ($\lambda_{318\ nm}$) for aztreonam, $-14{,}500\ M^{-1} \cdot cm^{-1}$ ($\lambda_{270\ nm}$) for ceftriaxone, $-2{,}500\ M^{-1} \cdot cm^{-1}$ ($\lambda_{227\ nm}$) for clavulanate, and $-7{,}280\ M^{-1} \cdot cm^{-1}$ ($\lambda_{298\ nm}$) for meropenem.

The D179Y substitution severely impaired the hydrolysis of ampicillin, aztreonam, and meropenem, since hydrolysis of these β-lactams by KPC-2 D179Y was not detectable at the highest enzyme concentration tested (10 μM) (Table 2). Accordingly, KPC-2 D179Y did not confer resistance to aztreonam and meropenem (Table 1). For ampicillin, the production of KPC-2 D179Y led to a 4-fold increase in the MIC, suggesting that the enzyme retained residual activity which could not be detected due to the low sensitivity of the *in vitro* assay for this antibiotic ($\Delta\varepsilon = -500\ M^{-1} \cdot cm^{-1}$ at $\lambda_{235\ nm}$).

The D179Y substitution led to a >900-fold reduction in the kinetic constant k_{cat} for hydrolysis of ceftazidime, ceftriaxone, and the chromogenic cephalosporin CENTA. Binding of these drugs to the β-lactamase did not appear to be impaired, since the D179Y substitution also led to a large reduction in the kinetic constant K_m for ceftazidime (>30-fold) and ceftriaxone (>500-fold), whereas that of CENTA was not significantly altered. Overall, the D179Y substitution had a moderate impact on the catalytic efficacy (k_{cat}/K_m) of ceftazidime hydrolysis (a 50-fold decrease) due to a partially compensatory effect of the large decrease in k_{cat} (>1,000-fold) by a decrease in K_m (>30-fold). Of note, this modification of the kinetic parameters did not prevent the expression of ceftazidime resistance (MIC, >128 μg/ml). The results obtained with ceftriaxone were qualitatively similar, although for this drug, the partial compensation of the decreases in k_{cat} and K_m was associated with a lower level of resistance (MIC, 32 μg/ml).

Impact of the D179Y substitution on inhibition of KPC-2 by avibactam. The rate constant for the carbamylation reaction (k_2/K_i) was evaluated based on the inhibition of CENTA hydrolysis (100 μM) by avibactam at 37°C in MES (100 mM [pH 6.4]) for a two-step reaction (Fig. 1), as previously described (11, 15). The D179Y substitution led to an approximately 70,000-fold reduction in the carbamylation rate constant k_2/K_i. Thus, resistance to the ceftazidime-avibactam combination conveyed by the D179Y substitution is accounted for by a drastic reduction in the efficacy of avibactam coupled to substantial residual activity for ceftazidime hydrolysis.

Selection of mutants coresistant to imipenem and the ceftazidime-avibactam combination. *E. coli* TOP10 harboring pTRC-99kΩbla_{KPC-2} (ca. 6×10^9 CFU) was plated on MH agar containing avibactam (4 μg/ml), ceftazidime (8 μg/ml), and imipenem (0.12 to 32 μg/ml). Mutants resistant to the triple combination were not obtained (frequency, $<2 \times 10^{-10}$).

The D179Y substitution leads to decreased hydrolysis of meropenem and imipenem by the enzyme and consequently does not confer resistance to those substrates once produced in *E. coli* (see above, Table 1). To assess whether secondary mutations could restore resistance to carbapenems, derivatives of *E. coli* TOP10 producing KPC-2 D179Y were selected on MH agar containing imipenem (8 μg/ml). Mutants were obtained at a frequency of 2×10^{-9}. Sequencing of the bla_{KPC-2} gene from 8 out of 8 mutants

TABLE 2 Impact of $D^{179}Y$ substitution on kinetic parameters of KPC-2 for hydrolysis of β-lactams

Kinetic parameter by β-lactam	Results[b]	
	KPC-2	KPC-2 $D^{179}Y$
Ampicillin		
k_{cat} (s^{-1})	200 ± 10	NA[a]
K_m (μM)	200 ± 40	NA
k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	$(1.0 \pm 0.2) \times 10^6$	NA
CENTA		
k_{cat} (s^{-1})	100 ± 10	0.11 ± 0.01
K_m (μM)	32 ± 8	23 ± 3
k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	$(3.1 \pm 0.8) \times 10^6$	$(4.8 \pm 0.6) \times 10^3$
Ceftazidime		
k_{cat} (s^{-1})	>1.4	$(1.3 \pm 0.2) \times 10^{-3}$
K_m (μM)	>600	19 ± 4
k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	$(3.7 \pm 0.1) \times 10^3$	70 ± 20
Ceftriaxone		
k_{cat} (s^{-1})	>25	$(7.0 \pm 0.1) \times 10^{-4}$
K_m (μM)	>100	0.20 ± 0.03
k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	$(2.5 \pm 0.1) \times 10^5$	$(3.5 \pm 0.5) \times 10^3$
Aztreonam		
k_{cat} (s^{-1})	>350	NA
K_m (μM)	$>5 \times 10^3$	NA
k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	$(6.9 \pm 0.3) \times 10^4$	NA
Meropenem		
k_{cat} (s^{-1})	1.8 ± 0.2	NA
K_m (μM)	27 ± 9	NA
k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	$(6.7 \pm 2.3) \times 10^4$	NA
Imipenem		
k_{cat} (s^{-1})	48 ± 5	NA
K_m (μM)	66 ± 21	NA
k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	$(7.3 \pm 2.4) \times 10^5$	NA
Clavulanate		
k_{cat} (s^{-1})	5.2 ± 0.7	NA
K_m (μM)	36 ± 4	NA
k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)	$(1.4 \pm 0.3) \times 10^5$	NA

[a]NA, not applicable, as hydrolysis of β-lactams (100 μM, except for aztreonam at 1,000 μM) was not detected at the highest β-lactamase concentration tested (10 μM). Under these conditions, the lower limits of detection correspond to a turnover of $<2 \times 10^{-3} \cdot s^{-1}$, $<2.6 \times 10^{-3} \cdot s^{-1}$, $<1.5 \times 10^{-4} \cdot s^{-1}$, $<1.5 \times 10^{-4} \cdot s^{-1}$, and $<4 \times 10^{-4} \cdot s^{-1}$ for ampicillin, aztreonam, meropenem, imipenem, and clavulanate, respectively.

[b]Data are means $\pm$ standard errors of the mean (SEM).

revealed a reversion to the wild-type sequence. These results indicate that the alteration of KPC-2 conferring coresistance to the ceftazidime-avibactam combination and to imipenem are not readily selected *in vitro*.

Conclusions. Here, we show that a single amino acid substitution in KPC-2 ($D^{179}Y$) is sufficient to prevent inhibition of the β-lactamase by avibactam, leading to resistance to the ceftazidime-avibactam combination. In contrast, previous analyses based on site-directed mutagenesis have shown that reduced inhibition by avibactam due to a modification of position 132 of KPC-2 ($N^{132}G$) does not confer resistance to the ceftazidime-avibactam combination, since the hydrolysis of ceftazidime is drastically reduced (11). Extensive analysis of the Ω-loop of KPC-2 by site-directed mutagenesis (positions 164, 167, 169, and 179) has identified only a few substitutions that result in resistance to the ceftazidime-avibactam combination (R^{164} replaced by A or P, and D^{179} replaced by A, Q, or N) (10). The underlying mechanism was proposed to involve stabilizing interactions (e.g., hydrogen bonds) of ceftazidime within the KPC-2 active

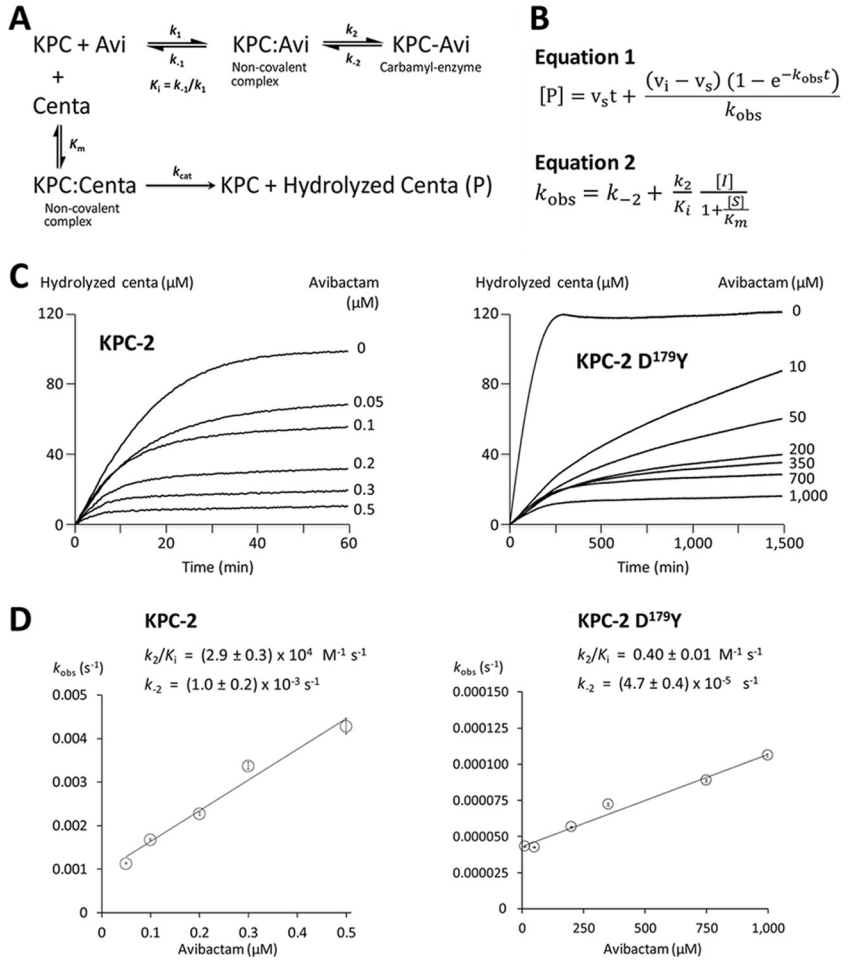

FIG 1 Kinetics of KPC-2 and KPC-2 D179Y inhibition by avibactam (Avi). (A) Reaction schemes. (B) Equations for rate constants. v_i and v_s are the initial and steady-state (inhibited) velocities of the reactions, respectively. [I], [P], and [S] are the concentrations of avibactam, hydrolyzed CENTA, and CENTA, respectively. K_m is the kinetic parameter for hydrolysis of CENTA in the absence of avibactam. *t*, time. (C) Inhibition of CENTA hydrolysis by increasing concentrations of avibactam. Values of k_{obs} were obtained by fitting equation 1 to progress curves. (D) Parameters k_2/K_i and k_{-2} were determined by fitting equation 2 to the data. Error bars shown are ± standard error of the mean (SEM) from the fit to k_{obs}.

site that prevent avibactam from binding to and inhibiting the β-lactamase (10). The mechanism described in reference 10 is clearly distinct from the impaired carbamylation by avibactam reported for the D179Y substitution in the current study.

The D179Y substitution was recently shown to be responsible for the acquisition of ceftazidime-avibactam resistance under treatment in *K. pneumoniae* isolates producing the closely related enzyme KPC-3 (8, 16). The fact that a single mutation is sufficient for impaired inhibition of KPC β-lactamases by avibactam indicates that the emergence of resistance under treatment may jeopardize the efficacy of the ceftazidime-avibactam combination in the future (17). To date, the emergence of resistance was documented in a single study in ca. 8% of the patients infected with carbapenem-resistant *Enterobacteriaceae* and treated with the ceftazidime-avibactam combination (8).

The D179Y substitution abolished the hydrolysis of meropenem and aztreonam by KPC-2 and improved inhibition by clavulanate (Table 2). Thus, these β-lactams and classical β-lactamase inhibitors might provide therapeutic alternatives in case of the emergence of ceftazidime-avibactam resistance. It remains to be determined whether such alternatives could also exist for other modifications of KPC enzymes leading to ceftazidime-avibactam resistance. This might be the case since the mutations selected

under treatment (8) also led to large decreases in the MICs of carbapenems (18). Similarly, *in vitro* selection for resistance to the ceftazidime-avibactam combination in KPC-3-producing *K. pneumoniae* and *Enterobacter cloacae* strains led to large decreases in the MICs of carbapenems in the majority of the mutants (19). Our attempts to select mutants resistant to the triple combination of ceftazidime, imipenem, and avibactam were unsuccessful. Our selection for imipenem-resistant derivatives of the mutant producing KPC-2 $D^{179}Y$ led to reversion to the wild-type sequence at position 179 and to susceptibility to the ceftazidime-avibactam combination. Recently, Shields et al. also reported such reversions, but resistance to carbapenems and ceftazidime-avibactam has been obtained by multistep exposure (≥4 weeks) of ceftazidime-avibactam-resistant *K. pneumoniae* strains to meropenem, mainly due to modifications of the porin OmpK36 (20).

Carbapenems and classical β-lactamase inhibitors may provide alternatives for use against variants with impaired inhibition by avibactam, as previously observed for substitutions in the β-lactamases from mycobacteria (21). These observations indicate, as previously discussed (21), that the formulation of β-lactamase inhibitors independently from a β-lactam partner would provide clinicians with access to potentially useful therapeutic regimens based on combining β-lactams with approved inhibitors.

ACKNOWLEDGMENT

Avibactam was provided by AstraZeneca.

We declare no conflicts of interest.

REFERENCES

1. Tzouvelekis LS, Markogiannakis A, Psichogiou M, Tassios PT, Daikos GL. 2012. Carbapenemases in *Klebsiella pneumoniae* and other *Enterobacteriaceae*: an evolving crisis of global dimensions. Clin Microbiol Rev 25:682–707. https://doi.org/10.1128/CMR.05035-11.
2. Grundmann H, Glasner C, Albiger B, Aanensen DM, Tomlinson CT, Andrasevic AT, Canton R, Carmeli Y, Friedrich AW, Giske CG, Glupczynski Y, Gniadkowski M, Livermore DM, Nordmann P, Poirel L, Rossolini GM, Seifert H, Vatopoulos A, Walsh T, Woodford N, Monnet DL, European Survey of Carbapenemase-Producing Enterobacteriaceae Working Group. 2017. Occurrence of carbapenemase-producing *Klebsiella pneumoniae* and *Escherichia coli* in the European survey of carbapenemase-producing *Enterobacteriaceae* (EuSCAPE): a prospective, multinational study. Lancet Infect Dis 17:153–163. https://doi.org/10.1016/S1473-3099(16)30257-2.
3. Monaco M, Giani T, Raffone M, Arena F, Garcia-Fernandez A, Pollini S, Network Eu S-I, Grundmann H, Pantosti A, Rossolini GM. 2014. Colistin resistance superimposed to endemic carbapenem-resistant *Klebsiella pneumoniae*: a rapidly evolving problem in Italy, November 2013 to April 2014. Euro Surveill 19:pii=20939. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20939.
4. Drawz SM, Bonomo RA. 2010. Three decades of beta-lactamase inhibitors. Clin Microbiol Rev 23:160–201. https://doi.org/10.1128/CMR.00037-09.
5. Papp-Wallace KM, Bonomo RA. 2016. New beta-lactamase inhibitors in the clinic. Infect Dis Clin North Am 30:441–464. https://doi.org/10.1016/j.idc.2016.02.007.
6. Sader HS, Castanheira M, Flamm RK. 2017. Antimicrobial activity of ceftazidime-avibactam against Gram-negative bacteria isolated from patients hospitalized with pneumonia in U.S. medical centers, 2011 to 2015. Antimicrob Agents Chemother 61:e02083-16. https://doi.org/10.1128/AAC.02083-16.
7. Lahiri SD, Bradford PA, Nichols WW, Alm RA. 2016. Structural and sequence analysis of class A beta-lactamases with respect to avibactam inhibition: impact of Ω-loop variations. J Antimicrob Chemother 71:2848–2855. https://doi.org/10.1093/jac/dkw248.
8. Shields RK, Potoski BA, Haidar G, Hao B, Doi Y, Chen L, Press EG, Kreiswirth BN, Clancy CJ, Nguyen MH. 2016. Clinical outcomes, drug toxicity and emergence of ceftazidime-avibactam resistance among patients treated for carbapenem-resistant *Enterobacteriaceae* infections. Clin Infect Dis 63:1615–1618. https://doi.org/10.1093/cid/ciw636.
9. Shields RK, Chen L, Cheng S, Chavda KD, Press EG, Snyder A, Pandey R, Doi Y, Kreiswirth BN, Nguyen MH, Clancy CJ. 2017. Emergence of ceftazidime-avibactam resistance due to plasmid-borne bla_{KPC-3} mutations during treatment of carbapenem-resistant *Klebsiella pneumoniae* infections. Antimicrob Agents Chemother 61:e02097-16. https://doi.org/10.1128/AAC.02097-16.
10. Winkler ML, Papp-Wallace KM, Bonomo RA. 2015. Activity of ceftazidime/avibactam against isogenic strains of *Escherichia coli* containing KPC and SHV beta-lactamases with single amino acid substitutions in the Ω-loop. J Antimicrob Chemother 70:2279–2286. https://doi.org/10.1093/jac/dkv094.
11. Ourghanlian C, Soroka D, Arthur M. 2017. Inhibition by avibactam and clavulanate of the β-lactamases KPC-2 and CTX-M-15 harboring the substitution $N^{132}G$ in the conserved SDN motif. Antimicrob Agents Chemother 61:e02510-16. https://doi.org/10.1128/AAC.02510-16.
12. Clinical and Laboratory Standards Institute. 2015. Methods for dilution susceptibility tests for bacteria that grow aerobically; approved standard, 10th ed. CLSI document M7-A8. Clinical and Laboratory Standards Institute, Wayne, PA.
13. Bebrone C, Moali C, Mahy F, Rival S, Docquier JD, Rossolini GM, Fastrez J, Pratt RF, Frere JM, Galleni M. 2001. CENTA as a chromogenic substrate for studying beta-lactamases. Antimicrob Agents Chemother 45:1868–1871. https://doi.org/10.1128/AAC.45.6.1868-1871.2001.
14. Frère JM, Dormans C, Duyckaerts C, De Graeve J. 1982. Interaction of beta-iodopenicillanate with the beta-lactamases of *Streptomyces albus* G and *Actinomadura* R39. Biochem J 207:437–444. https://doi.org/10.1042/bj2070437.
15. Ehmann DE, Jahic H, Ross PL, Gu RF, Hu J, Kern G, Walkup GK, Fisher SL. 2012. Avibactam is a covalent, reversible, non-beta-lactam beta-lactamase inhibitor. Proc Natl Acad Sci U S A 109:11663–11668. https://doi.org/10.1073/pnas.1205073109.
16. Shields RK, Clancy CJ, Hao B, Chen L, Press EG, Iovine NM, Kreiswirth BN, Nguyen MH. 2015. Effects of *Klebsiella pneumoniae* carbapenemase subtypes, extended-spectrum beta-lactamases, and porin mutations on the *in vitro* activity of ceftazidime-avibactam against carbapenem-resistant *K. pneumoniae*. Antimicrob Agents Chemother 59:5793–5797. https://doi.org/10.1128/AAC.00548-15.
17. Spellberg B, Bonomo RA. 2016. Ceftazidime-avibactam and carbapenem-resistant *Enterobacteriaceae*: "we're gonna need a bigger boat." Clin Infect Dis 63:1619–1621. https://doi.org/10.1093/cid/ciw639.
18. Haidar G, Clancy CJ, Shields RK, Hao B, Cheng S, Hong Nguyen M. 2017. Mutations in bla_{KPC-3} that confer ceftazidime-avibactam resistance en-

code novel KPC-3 variants that function as extended-spectrum β-lactamases. Antimicrob Agent Chemother, 61:e02534-16. https://doi.org/10.1128/AAC.02534-16.
19. Livermore DM, Warner M, Jamrozy D, Mushtaq S, Nichols WW, Mustafa N, Woodford N. 2015. *In vitro* selection of ceftazidime-avibactam resistance in *Enterobacteriaceae* with KPC-3 carbapenemase. Antimicrob Agents Chemother 59:5324–5330. https://doi.org/10.1128/AAC.00678-15.
20. Shields RK, Nguyen MH, Press EG, Chen L, Kreiswirth BN, Clancy CJ. 2017. *In vitro* selection of meropenem resistance among ceftazidime-avibactam resistant, meropenem susceptible *Klebsiella pneumoniae* isolates with variant KPC-3 carbapenemases. Antimicrob Agents Chemother, 61:e00079-17. https://doi.org/10.1128/AAC.00079-17.
21. Soroka D, Ourghanlian C, Compain F, Fichini M, Dubée V, Mainardi J, Hugonnet J, Arthur M. 2017. Inhibition of β-lactamases of mycobacteria by avibactam and clavulanate. J Antimicrob Chemother 72:1081–1088. https://doi.org/10.1093/jac/dkw546.