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Exploring the Landscape of Diazabicyclooctane (DBO) Inhibition: Avibactam Inactivation of PER-2 β-Lactamase

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ABSTRACT PER β-lactamases are an emerging family of extended-spectrum β-lactamases (ESBL) found in Gram-negative bacteria. PER β-lactamases are unique among class A enzymes as they possess an inverted omega (Ω) loop and extended B3 β-strand. These singular structural features are hypothesized to contribute to their hydrolytic profile against oxyimino-cephalosporins (e.g., cefotaxime and ceftazidime). Here, we tested the ability of avibactam (AVI), a novel non-β-lactam β-lactamase inhibitor to inactivate PER-2. Interestingly, the PER-2 inhibition constants (i.e., k_2/K = $2 \times 10^3 \pm 0.1 \times 10^3$ M^{-1} s^{-1}, where k_2 is the rate constant for acylation (carbamylation) and K is the equilibrium constant) that were obtained when AVI was tested were reminiscent of values observed testing the inhibition by AVI of class C and D β-lactamases (i.e., k_2/K range of $\approx 10^3$ M^{-1} s^{-1}) and not class A β-lactamases (i.e., k_2/K range, 10^4 to 10^5 M^{-1} s^{-1}). Once AVI was bound, a stable complex with PER-2 was observed via mass spectrometry (e.g., 31,389 ± 3 atomic mass units [amu] → 31,604 ± 3 amu for 24 h). Molecular modeling of PER-2 with AVI showed that the carbonyl of AVI was located in the oxyanion hole of the β-lactamase and that the sulfate of AVI formed interactions with the β-lactam carboxylate binding site of the PER-2 β-lactamase (R220 and T237). However, hydrophobic patches near the PER-2 active site (by Ser70 and B3-B4 β-strands) were observed and may affect the binding of necessary catalytic water molecules, thus slowing acylation (k_2/K) of AVI onto PER-2. Similar electrostatics and hydrophobicity of the active site were also observed between OXA-48 and PER-2, while CTX-M-15 was more hydrophilic. To demonstrate the ability of AVI to overcome the enhanced cephalosporinase activity of PER-2 β-lactamase, we tested different β-lactam–AVI combinations. By lowering MICs to ≤2 mg/liter, the ceftaroline-AVI combination could represent a favorable therapeutic option against *Enterobacteriaceae* expressing bla_{PER-2}. Our studies define the inactivation of the PER-2 ESBL by AVI and suggest that the biophysical properties of the active site contribute to determining the efficiency of inactivation.

KEYWORDS avibactam, beta-lactamases, beta-lactams

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PER, a class A extended-spectrum β-lactamase (ESBL), is an emerging resistance determinant within Gram-negative bacteria. Presently, PER ESBLs are found in *Acinetobacter* spp., *Escherichia coli*, and *Klebsiella pneumoniae*. Notably, PER β-lactamases are frequently detected in South America (Argentina and Uruguay), as well as in regions of the Middle East. PER-2 is the second most prevalent ESBL (after the pandemic CTX-M) found in Argentina, accounting for up to 10% and 5% of the oxyimino-cephalosporin resistance in *K. pneumoniae* and *E. coli*, respectively (1, 2).

PER-2 shares 86% amino acid sequence identity with PER-1 and readily hydrolyzes oxyimino-cephalosporins, with high catalytic efficiencies (k_{cat}/K_m) for both cefotaxime and ceftazidime (CAZ), the latter being ~22-fold higher in PER-2 than in PER-1. PER β-lactamases are also inhibited by mechanism-based inhibitors such as clavulanate and tazobactam (3, 4). Recently, the X-ray crystal structure of PER-2 was determined at 2.2 Å (5). Among the most relevant aspects in the structure, PER-2 is defined by the presence of a singular *trans* bond between Ambler positions 166 and 167 that generates an inverted omega (Ω) loop. Together with an expanded β3-β4 loop, the active-site cavity created is enlarged 2-fold compared to the cavities of other class A β-lactamases. This expansion may contribute to the efficient hydrolysis of β-lactams like the oxyimino-cephalosporins. Notably, a hydrogen bond network connecting Ser70-Gln69-water-Thr237-Arg220 exists that might also be important for the enhanced activity and inhibition of the enzyme (5).

Avibactam (AVI) is a novel bridged diazabicyclooctane (DBO) non-β-lactam β-lactamase inhibitor (BLI) (6) that reversibly inactivates most Ambler class A and C β-lactamases. Combinations of ceftazidime (CAZ)-AVI, ceftaroline (CPT)-AVI, and aztreonam (ATM)-AVI were shown to be effective against aerobic Gram-negative bacilli such as *Enterobacteriaceae* and *Pseudomonas aeruginosa* producing these β-lactamases (7–12). AVI is responsible for this expanded microbiological activity. In addition to improved affinity, upon deacylation, AVI undergoes a recyclization via ring closure (i.e., regenerating the intact avibactam, albeit at a low rate in TEM-1 and SHV-1) and may acylate other β-lactamases (6, 22). In contrast, the inactivation of β-lactamases by a β-lactamase inhibitor such as tazobactam, sulbactam, or clavulanic acid involves a nucleophilic attack by the active-site serine on the β-lactam's amide bond, acylation of the enzyme, subsequent rearrangement steps resulting in imine or enamine intermediates, and a transient or long-term inhibition of the enzyme (17). In spite of these differences, AVI shares some mechanistic similarities with other inhibitors, such as a carbonyl carbon, acylation of Ser70, and accommodation of the carbonyl oxygen in the oxyanion hole for stabilization of the transition state (14). Several crystallographic structures of serine β-lactamases have been solved in complex with AVI, supporting these mechanistic characteristics (14, 15, 18, 19).

Interestingly, amino acid substitutions or deletions within the Ω loop of class A and C β-lactamases result in β-lactamases with enhanced turnover/binding of CAZ, reducing the activity of CAZ-AVI in bacteria (16, 20, 21, 31, 32). Given the unique Ω loop of PER-2, we investigated the combinations of CAZ-AVI, CPT-AVI, and ATM-AVI to determine their activity against isolates expressing PER β-lactamases, and in particular PER-2, which is an important plasmid-borne acquired ESBL. The goals of these studies are to assess the ability of AVI to inhibit PER-2, to restore susceptibility to clinical strains when it is combined with CAZ, CPT, or ATM, and to probe the mechanism of inhibition.

RESULTS AND DISCUSSION

AVI inhibition of PER-2 β-lactamase. The apparent inhibition constant ($K_{i\ app}$) for AVI, determined by plotting $1/v_0$, where v_0 is initial velocity, versus concentration of the inhibitor, [AVI], was 20 ± 3 μM. This determination was ~1,000-fold greater than the value reported for wild-type SHV-1 (22) and 17-fold greater than that for KPC-2 (30) (Table 1) although the value was similar to the $K_{i\ app}$ displayed by the cephalosporinase CMY-2 (23) (Table 1).

Progress curves measuring the rate of AVI inhibition were obtained for PER-2 by using increasing concentrations of AVI (range, 2 to 32 μM) as a competitive inhibitor of

TABLE 1 Comparison of enzyme inhibition kinetic parameters of avibactam toward PER-2 and other β-lactamases

β-Lactamase	Value for the parameter with avibactam				Reference(s)
	K_{iapp} (μM)	k_2/K (M^{-1} s^{-1})	k_{off} (s^{-1})	$t_{1/2}$ (min)	
PER-2	20 ± 3	(2.2 ± 0.1) × 10^3	4.0 × 10^{-4}	29 ± 2	
KPC-2	1.2 ± 0.1	1.7 × 10^4	1.1 × 10^{-4}	82 ± 6	13, 30
SHV-1	0.022 ± 0.002	6 × 10^4			22
CTX-M-15		(1.3 ± 0.1) × 10^5	(3 ± 1) × 10^{-4}	40 ± 10	13
TEM-1		(1.6 ± 0.1) × 10^5	(8 ± 4) × 10^{-4}	16 ± 8	6
OXA-10		(1.1 ± 0.1) × 10^1	<1.6 × 10^{-6}	>7,200	13
OXA-48		(1.4 ± 0.1) × 10^3	(1.2 ± 0.4) × 10^{-5}	1,000 ± 300	13
CMY-2	26 ± 3	(4.9 ± 0.5) × 10^4	(3.7 ± 0.4) × 10^{-4}	31 ± 4	23
E. cloacae P99 AmpC		(5.1 ± 0.1) × 10^3	(3.8 ± 0.2) × 10^{-5}	300 ± 20	13
P. aeruginosa PAO1 AmpC (PDC-1)		(2.9 ± 0.1) × 10^3	(1.9 ± 0.6) × 10^{-3}	6 ± 2	13

nitrocefin (NCF) hydrolysis (Fig. 1a). Progress curves were fit to obtain values for the observed inhibition rate constant (k_{obs}), as described in the Materials and Methods section, and k_{obs} values were plotted against AVI concentrations (Fig. 1b) to obtain k_2/K, where k_2 is the rate constant for acylation (carbamylation) and K is the equilibrium constant. For PER-2, acylation and encounter complex binding for AVI occur with a k_2/K value of 2,200 ± 100 M^{-1} s^{-1}. Comparative analysis with other serine β-lactamases (Table 1) showed that the ESBL PER-2 displayed acylation rate constants similar to those of class C β-lactamases from *Enterobacter cloacae* (P99) and *Pseudomonas aeruginosa* (PAO1) and the carbapenem-hydrolyzing class D (CHDL) OXA-48 (13) (k_2/K values in the range of 10^3 M^{-1} s^{-1}). These values are 100-fold less than k_2/K values for other class A β-lactamases, such as TEM-1 and CTX-M-15 (6, 13), and 10-fold less than those for KPC-2 (30). Also, acylation efficiency for AVI is 20-fold less for PER-2 than for the class C β-lactamase CMY-2 (23) although it is a 180-fold higher than that for OXA-10, another CHDL not related to the OXA-48 group (13).

Recovery of PER-2 activity after inhibition by AVI was measured to obtain the off-rate constant, k_{off} (Fig. 2). We determined k_{off} to be 0.0004 s^{-1} with a half-time ($t_{1/2}$) of 29 min (Table 1). The off-rate for PER-2 was comparable to the values for other class A β-lactamases, including the ESBL CTX-M-15, and to that of the class C enzyme CMY-2; it was between 34- and 250-fold greater than that of class D β-lactamases OXA-10 and OXA-48, 10-fold greater than that of P99 AmpC, but 5-fold lower than that of PAO1 AmpC (Table 1). As anticipated, the half-life for the PER-2–AVI combination was similar to the values for CMY-2 and CTX-M-15 and nearly three times shorter than the half-life for KPC-2, but this combination recovered much faster than class D enzymes.

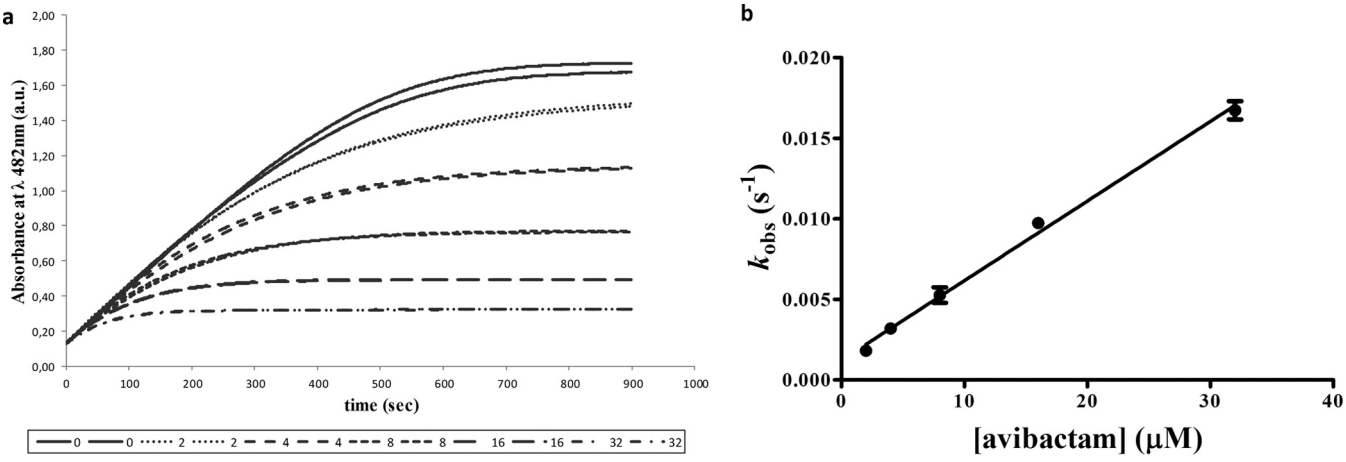

FIG 1 (a) Progress curves using increasing concentrations of avibactam (AVI) to inhibit nitrocefin hydrolysis by PER-2 in duplicate. (b) k_{obs} values obtained from the progress curves in panel a, plotted against AVI concentration. These values were used to obtain k_2/K. a.u., arbitrary units.

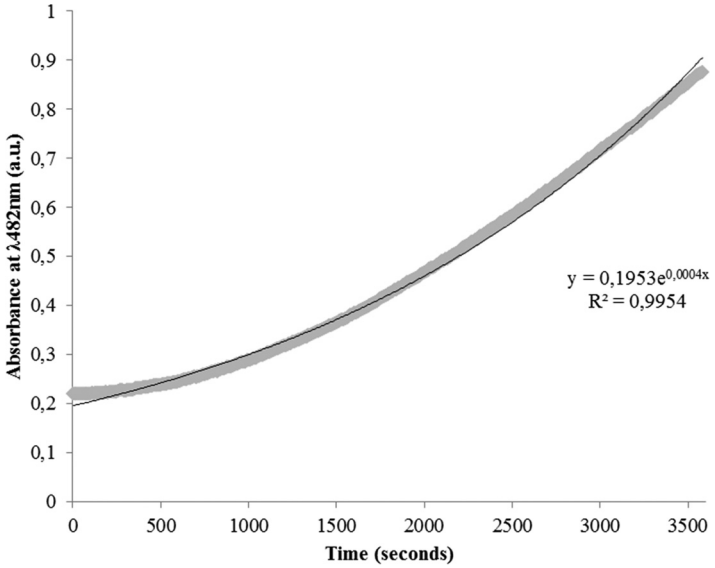

FIG 2 Recovery of nitrocefin hydrolysis activity by PER-2 after inhibition by AVI fit to a single exponential equation to obtain $k_{off} = 0.0004\ s^{-1}$.

AVI forms a stable complex with PER-2 for 24 h. Electrospray ionization mass spectrometry (ESI-MS) of PER-2 inactivated with AVI for 5 min and 24 h revealed that AVI was not modified (i.e., desulfated) or hydrolyzed by PER-2 under these conditions (Fig. 3). The measured molecular mass of PER-2 was 31,339 ± 3 atomic mass units (amu). Inactivation of PER-2 with AVI shifted the peak by +265 amu, which is consistent with the addition of an unmodified AVI to the PER-2 β-lactamase. Most class A and C

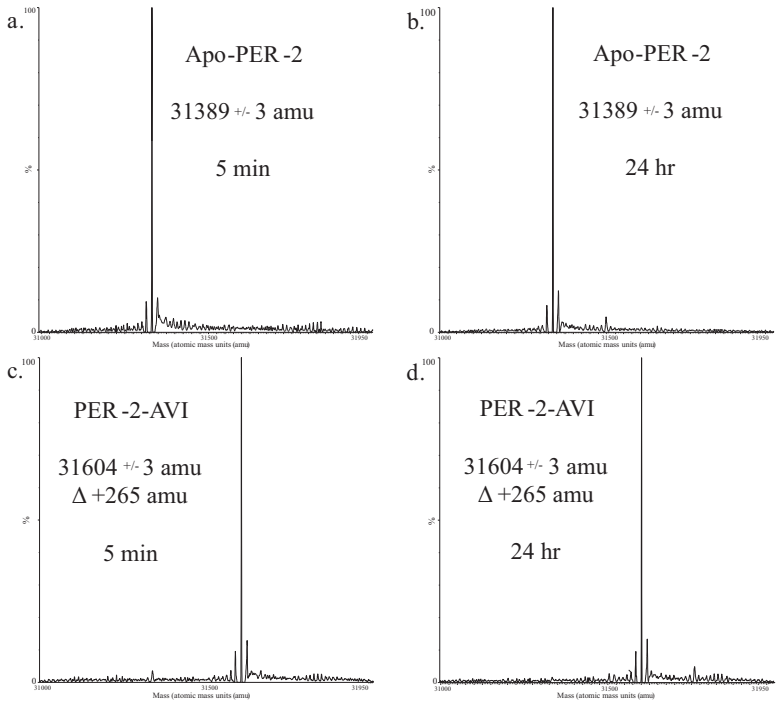

FIG 3 (a) Mass spectrometry of the PER-2 β-lactamase (error ± 3 atomic mass units [amu]). The mass of apo PER-2 is 31,339 ± 3 amu. (b) Mass spectrometry of PER-2 β-lactamase inactivated by AVI at a 1:1 ratio for 5 min. (The PER-2 concentration was 20 μM). The mass of the AVI-inactivated PER-2 β lactamase is 31,604 ± 3 amu. (c) Mass spectrometry of apo PER-2 inactivated at room temperature for 24 h. (d) Mass spectrometry of PER-2 inactivated by AVI for 24 h. Note the continued presence of acylated PER-2.

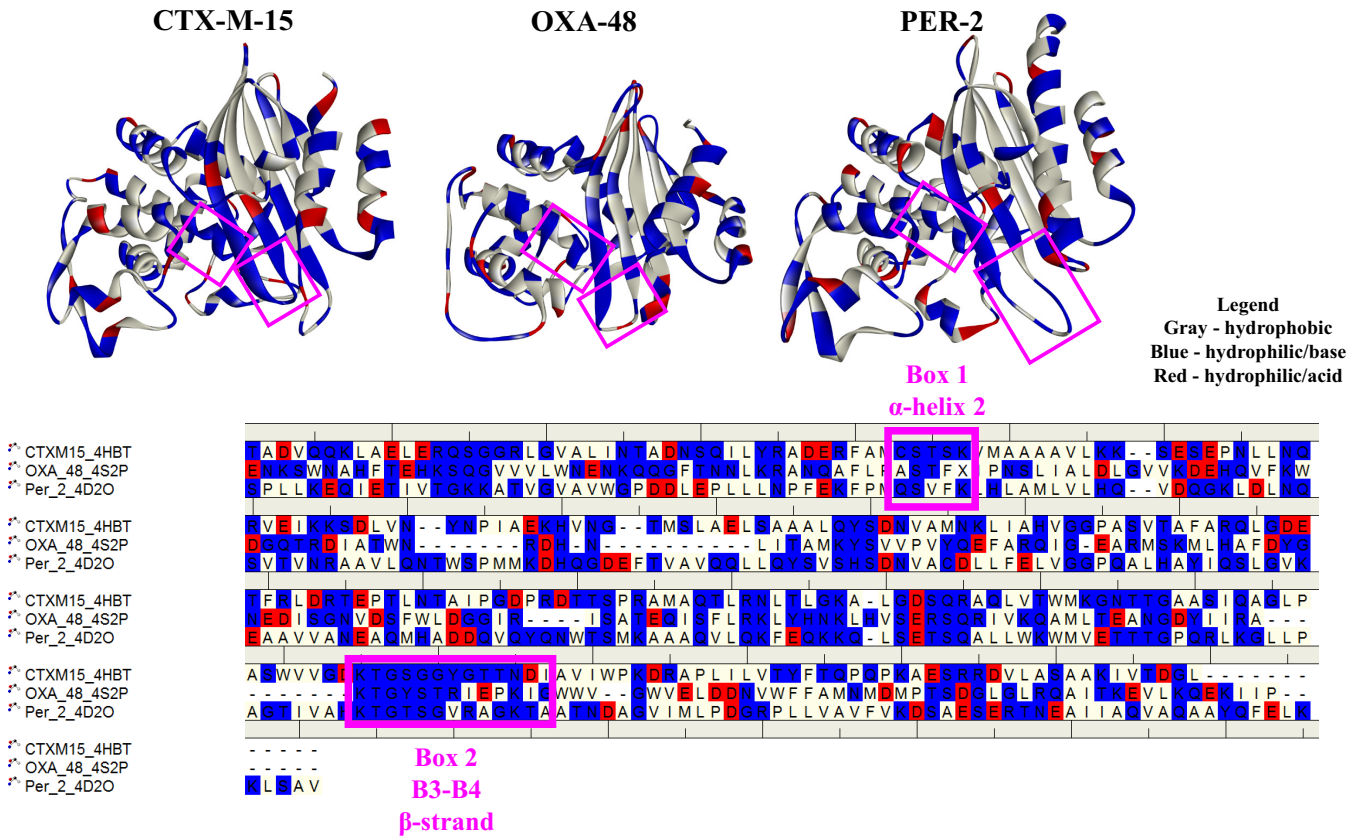

FIG 4 Molecular modeling revealing the overall electrostatic field of PER-2, OXA-48, and CTX-M-15. The catalytic sites (Box 1 and Box 2) reveal that PER-2 and OXA-48 possess more hydrophobic residues. Conversely, the catalytic site of CTX-M-15 is more hydrophilic than that of the other two enzymes.

serine β-lactamases form stable acyl-enzyme complexes with unmodified AVI. However, KPC-2 is the exception as AVI desulfates upon reaction with KPC-2 and is subsequently hydrolyzed (13).

Mechanistic insights revealed from PER-2 AVI modeling. To gain insights into the basis of AVI inhibition of PER-2, the apo enzymes of PER-2, CTX-M-15, and OXA-48 were compared, and Michaelis complexes were generated with AVI in the active sites of PER-2 and CTX-M-15. The question we sought to address was whether structural differences or similarities exist between CTX-M-15, OXA-48, and PER-2 that could impact acylation by AVI. The magnitude of the k_2/K value for PER-2 with AVI (10^3 M^{-1} s^{-1}) more closely resembles the value with the class D β-lactamase OXA-48 (10^3 M^{-1} s^{-1}) than the value of another class A ESBL, CTX-M-15 (10^5 $M^{-1}s^{-1}$).

Using the crystallographic structures of PER-2 (PDB accession number 4D2O), CTX-M-15 (PDB accession number 4HBT), and OXA-48 (PDB accession number 4S2P), we first compared the apo enzymes (5, 15, 19). The lower acylation rate of AVI for class D OXA β-lactamases is hypothesized to be the result of the largely hydrophobic active sites found in OXAs, which directly impacts the binding of AVI (15). This hydrophobicity also likely affects the recruitment and binding of catalytic water molecules. Upon inspection of the apo structures using Discovery Studio software, OXA-48 was found to be the most hydrophobic, followed by CTX-M-15 and PER-2 (data not shown). However, examining the localized electrostatic field and hydrophobicity near the active sites of PER-2, CTX-M-15, and OXA-48 reveals important differences (Fig. 4). The catalytic site of CTX-M-15 is more hydrophilic than the sites of the other two enzymes. Two hydrophobic patches (near Ser70 and the B3-B4 β-strand loop and B4 β-strand) are present in OXA-48 and PER-2, and these may directly contribute to the similarity of the k_2/K

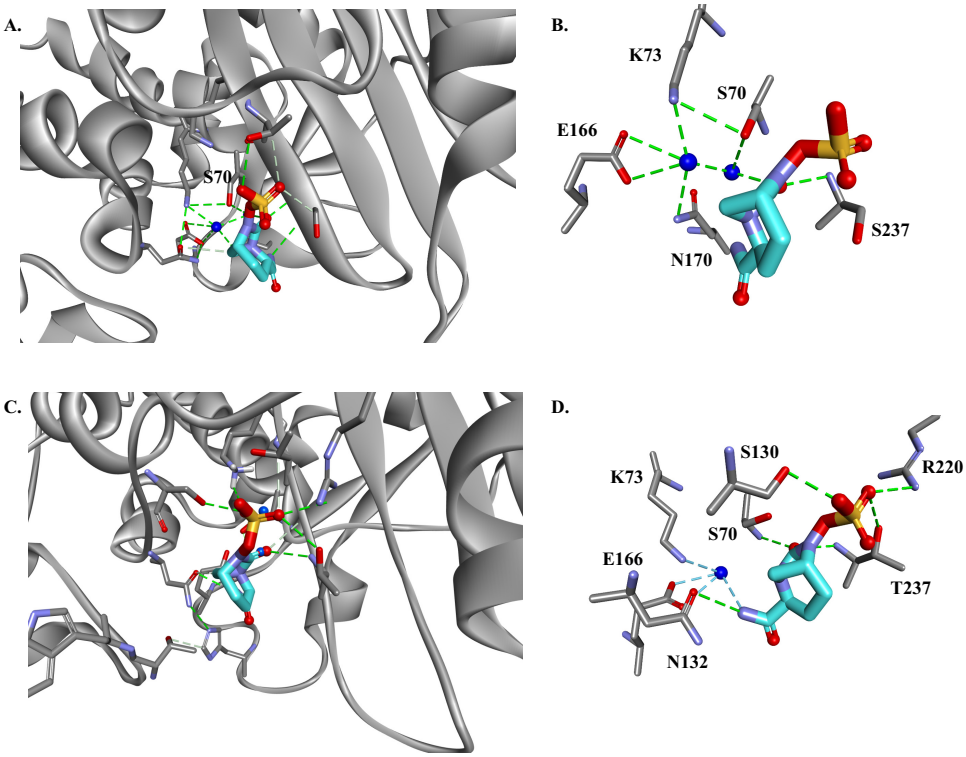

FIG 5 Michaelis-Menten complex of CTX-M-15 (A and B) and PER-2 (C and D) with AVI. Catalytic water molecules are represented by blue spheres, and hydrogen bonding interactions are presented as dashed green lines. The avibactam molecule is in cyan. Other atoms are in red (oxygen), blue (nitrogen), and yellow (sulfur).

values for AVI with PER-2 and OXA-48 (Fig. 4). These patches likely also impact catalytic water molecule networking (Fig. 5). On the other hand, CTX-M-15 possesses a more hydrophilic environment, creating a favorable environment for the water interactions. Indeed, the number of water molecules in the active site of CTX-M-15 is higher than the numbers in OXA-48 and PER-2.

The Michaelis complexes of AVI in CTX-M-15 and PER-2 were compared (Fig. 5). The catalytic waters present in the CTX-M-15 structure (PDB accession number 4HBT) are preserved in the active site of the Michaelis complex when AVI is modeled (Fig. 5A and B). One water molecule in particular is positioned favorably to activate Ser70 for AVI acylation with hydrogen bonding interactions with Ser70:Oγ and less than 3 Å from C-6 of AVI. The AVI carbonyl (:O2) is positioned in the oxyanion hole of CTX-M-15 and hydrogen bonded with Ser237. Lys73 is positioned within hydrogen bonding distance of Ser70. A second catalytic water molecule is held by Glu166 and Asn170 and is less than 2.8 Å from the catalytic serine. This model is suggestive of a favorable conformation for fast acylation of CTX-M-15 by AVI. Conversely, when the intact AVI is docked into the active site of PER-2, the catalytic waters are displaced (Fig. 5C and D). During the minimization, the water molecules are in an unfavorable position relative to Ser70 (4 Å away). Additionally, Lys73 is more than 5 Å from the catalytic serine as well. However, the carbonyl of AVI is well positioned into the oxyanion hole, and the compound forms hydrogen bonds with Ser130, Asn132, and Arg220 and Thr237 (the latter two amino acids defining the carboxylate binding site) (Fig. 4D).

Antimicrobial susceptibility testing (AST): proof of principle. In order to validate our kinetic and modeling studies, we chose select isolates expressing bla_{PER-2} to test the efficacy of AVI inactivation of the PER-2 β-lactamase in bacterial cells. Given the importance of the ESBL profile, we tested AVI with three different partner β-lactams: CAZ, ATM, and CPT.

The results obtained from the susceptibility testing show that AVI, upon combination with CAZ, ATM, and CPT, effectively reduced the MIC values in the strains tested

TABLE 2 MICs for clinical isolates of *Enterobacteriaceae* producing PER-2 β-lactamase

Isolate	MIC for the isolate (μg/ml)[a]					
	CAZ	CAZ-AVI	CPT	CPT-AVI	AZT	AZT-AVI
Escherichia coli DH10B pBC SK(+)[b]	0.5	0.25	0.125	0.125	0.125	0.06
Citrobacter freundii 33587	512	8	16	0.5	512	2
Escherichia coli 8791	>512	2	32	0.25	>512	4
Escherichia coli 5706	512	4	16	0.25	256	4
Klebsiella pneumoniae 3863	512	4	>512	0.25	128	2
Klebsiella pneumoniae 34505	>512	16	32	0.5	>512	16
Klebsiella pneumoniae LUIS	512	8	32	0.5	256	2
Klebsiella pneumoniae 33822	>512	32	64	2	>512	16
Klebsiella pneumoniae 33002	>512	4	32	0.25	256	2
Klebsiella pneumoniae JMC	512	8	16	0.25	256	4
Klebsiella pneumoniae 33582	>512	32	64	1	>512	16

[a]CAZ, ceftazidime; AVI, avibactam; CPT, ceftaroline; AZT, aztreonam.
[b]This is a negative control with no β-lactamase.

(Table 2). Whereas AVI was successful in reducing the MIC for all three partner β-lactams, the combination with CPT was the only one that restored susceptibility in all isolates, reducing the MIC values (>2,000-fold reduction in μg/ml) compared to the MICs for the partner β-lactam alone.

Previous studies have reported a failure in the ability of CPT to inhibit ESBL-producing *Klebsiella* isolates (24). Therefore, our results are important because the BL-BLI combination CPT-AVI seems to be an initially attractive and promising option for ESBL producers like PER-2-producing *K. pneumoniae* isolates. Conversely, for CAZ and ATM combined with AVI, the intrinsically high-level resistance to the partner drugs (MIC of >512 μg/ml in some isolates) rendered the inhibitory activity of AVI insufficient for restoring full susceptibility levels, even when the MIC was lowered by 7 to 8 doubling dilutions (256-fold reduction in μg/ml) in these combinations. Some isolates, like *K. pneumoniae* isolates 34505, 33822, and 33582, still tested as resistant to CAZ-AVI according to the CLSI breakpoints.

The following PER-2 β-lactamase kinetic parameters were determined: for CAZ, $k_{cat}/K_m = 1.8 \pm 0.2\ \mu M^{-1}\ s^{-1}$ ($k_{cat} = 92 \pm 3\ s^{-1}$; $K_m = 51 \pm 3\ \mu M$); ATM, $k_{cat}/K_m = 0.40 \pm 0.04\ \mu M^{-1}\ s^{-1}$ ($k_{cat} = 1.0 \pm 0.1\ s^{-1}$; $K_m = 2.4 \pm 0.1\ \mu M$); CPT, $k_{cat}/K_m = 3.7 \pm 0.4\ \mu M^{-1}\ s^{-1}$ ($k_{cat} = 1.1 \pm 0.1\ s^{-1}$; $K_m = 0.30\ \mu M \pm 0.07$). Given these kinetic data, we postulate that the decreased efficacy of CAZ-AVI and ATM-AVI compared to that of CPT-AVI against isolates producing bla_{PER-2} is probably due to the penicillin-binding protein (PBP) affinity of CPT compared to those of CAZ and ATM. Studies are under way to define this difference.

Conclusions. In these studies, we characterize the kinetics of inactivation of PER-2 by AVI. The rate constants determined suggest that the kinetics of inactivation (k_2/K) is not as rapid as that of certain representative class A enzymes. Interestingly, the k_2/K value more closely approximates values seen with class D or C enzymes that have been previously reported. Based on the results of molecular modeling, we propose that the mechanism of inactivation of PER-2 and AVI is affected by hydrophobic patches present in the active site. Experiments are under way to replace these hydrophobic amino acids (e.g., Phe72, Val240, and Ala242) with hydrophilic residues to test our hypothesis. We predict that the k_2/K value for AVI will increase for these PER-2 variants. These structural considerations should be kept in mind as novel BLIs are designed.

As a test of our biochemical observations, we selected strains possessing PER-2 for susceptibility testing. The optimal combination to overcome the ESBL phenotype was CPT-AVI. This singular finding suggests that this combination may hold promise against highly drug-resistant non-metallo-β-lactamase-producing strains. Mechanistically, CPT PBP affinity may play a significant role in the efficacy of this combination. Studies are in progress to investigate these possibilities.

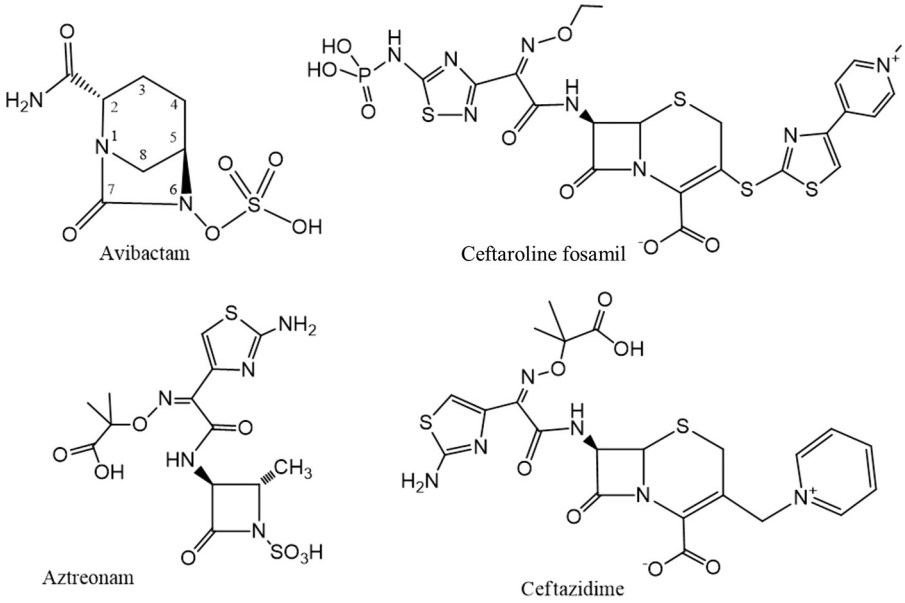

FIG 6 Chemical structures of avibactam, ceftazidime, ceftaroline fosamil, and aztreonam.

MATERIALS AND METHODS

Clinical isolates, reference strains, and plasmids. Clinical isolates of *Enterobacteriaceae* producing PER-2 β-lactamase belonged to a collection of strains already described in previous studies (1, 3): *K. pneumoniae* LUIS and JMC strains were isolated from a urinary tract infection (UTI) specimen at Clínica Lanús (Buenos Aires Province, Argentina) and from a blood culture at Sanatorio San Lucas (San Isidro, Buenos Aires Province, Argentina), respectively, in 1997; *E. coli* 8791 was isolated from a UTI specimen at Hospital Fernández (Buenos Aires City, Argentina) in 2000; *E. coli* 5706 and *K. pneumoniae* 3863 were recovered from UTI and bronchoalveolar lavage (BAL) specimens, respectively, at Hospital Muñiz (Buenos Aires City, Argentina) in 2004. The rest of the isolates employed were collected from urine cultures at Sanatorio San Lucas (San Isidro, Buenos Aires, Argentina), between 1999 and 2000.

E. coli TC9, a transconjugant strain harboring the pCf587 plasmid, was used as the source of bla_{PER-2} (3). *E. coli* Top10F′ (Invitrogen, USA) and *E. coli* BL21(DE3) (Novagen, USA) were hosts for transformation experiments and for β-lactamase expression and purification, respectively. Plasmid vectors pGEM-T Easy Vector (Promega, USA) and kanamycin-resistant pET28a(+) (Novagen, Germany) were employed for routine cloning experiments and for PER-2 β-lactamase purification, respectively. Recombinant clone *E. coli* BL-PER-2-BS harboring the pET/bla_{PER-2} plasmid was described previously (5).

Compounds. CAZ and ATM were purchased from Sigma-Aldrich and Research Products International Corp., respectively. AVI and CPT were acquired through an investigator-initiated trial with Actavis (now Allergan). Nitrocefin (NCF) was purchased from Becton-Dickinson and Oxoid. Chemical structures are shown in Fig. 6.

Antimicrobial susceptibility testing (AST). Determination of the MICs of CAZ, CAZ-AVI, CPT, CPT-AVI, ATM, and ATM-AVI was performed on clinical isolates of *Enterobacteriaceae* producing PER-2, according to the CLSI guidelines (25, 33). CAZ, CPT, and ATM were selected as the β-lactam partners to assess the degree of inhibition of β-lactamases by AVI. AVI was maintained at a constant concentration of 4 μg/ml. *E. coli* DH10B possessing pBC SK(+) served as the negative control.

Expression and purification of PER-2 β-lactamase. PER-2 was expressed and purified as previously described (5). Briefly, cultures of recombinant *E. coli* BL-PER-2-BS were grown in lysogeny broth (LB) with 30 μg/ml kanamycin to an optical density (OD) at a wavelength of 600 nm (λ_{600}) of 0.8, at which point induction of β-lactamase expression was initiated with the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and cultures were maintained at 37°C for 3 h. The cells were pelleted and subjected to mechanical disruption by sonication. The PER-2 β-lactamase was purified by nickel-based affinity chromatography using HisTrap HP affinity columns (GE Healthcare Life Sciences, USA), in an ÄKTA purifier (GE Healthcare, Uppsala, Sweden). The His tag on PER-2 was removed by cleavage with thrombin. The purity of PER-2 was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and protein concentration was determined by a bicinchoninic acid (BCA) protein quantitation assay (Pierce, Rockford, IL, USA).

Steady-state and inhibition kinetics. Steady-state kinetic parameters were determined using a T80 UV/visible light (VIS) spectrophotometer (PG Instruments, Ltd., United Kingdom) as previously reported (5, 13, 23). Briefly, each assay was performed in triplicate in 10 mM phosphate-buffered saline (PBS) at pH 7.4 at room temperature.

The steady-state kinetic parameters K_m and V_{max} were obtained by measuring initial rates as described previously (27), with nonlinear least-squares fit of the data (Henri Michaelis-Menten equation; equation 1) using GraphPad Prism, version 5.03 for Windows (GraphPad Software, San Diego, CA, USA):

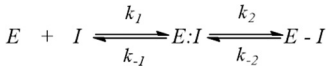

FIG 7 In this scheme, E represents PER-2, I represents AVI, E:I represents the Michaelis complex, and E − I represents the acyl enzyme.

$$v = (V_{max} \times [S])/(K_m + [S]) \quad (1)$$

where v is velocity and S is the substrate.

To measure steady-state kinetic parameters for CPT, the absorbance wavelength to measure hydrolysis was determined, and the extinction coefficient ($\Delta\varepsilon$) was calculated using Beer's law. Using 100 mM NaOH, complete hydrolysis of CPT was monitored; λ_{310} was selected for measurements with a $\Delta\varepsilon = -6{,}020\ cm^{-1}\ M^{-1}$.

The interaction of PER-2 with AVI is assumed to be represented by the scheme shown in Fig. 7, which was based on previous models proposed for other class A β-lactamases, excluding KPC-2 (6). The apparent K_i ($K_{i\ app}$) values can approximate the K_i (k_{-1}/k_1, where k_{-1} and k_1 are rate constants for reverse and forward reactions, respectively) of the inhibitor for β-lactamases that acylate very slowly; however, for β-lactamases that demonstrate a fast acylation rate, the $K_{i\ app}$ will approximate the K_m of the enzyme for the inhibitor (6). Determination of $K_{i\ app}$ was obtained using a direct competition assay under steady-state conditions and measuring initial velocities. The velocity (v_0) obtained after mixing corresponds to equation 2:

$$v_0 = (V_{max} \times [S])/\{K_m \times (1 + [I]/K_{i\,app}) + [S]\} \quad (2)$$

The enzyme concentration was kept at the nanomolar range, while the AVI concentration (I) was increased, and the inhibition was determined upon incubation with 110 μM NCF as the reporter substrate (S). Data were linearized by plotting the inverse initial steady-state velocity ($1/v_0$) against inhibitor concentration, [I]. K_{iapp} was determined by dividing the value for the y-intercept by the slope of the line and corrected to account for the concentration and affinity of nitrocefin (NCF) for the β-lactamase according to equation 3:

$$K_{i\,app\,(corrected)} = K_{i\,app\,(observed)}/(1 + [S]/K_m\ \mathrm{NCF}) \quad (3)$$

To determine k_2/K, progress curves were obtained by incubating PER-2 with increasing concentrations of AVI and maintaining NCF at 110 μM as the reporter substrate. Progress curves were subsequently fit to equation 4 to obtain k_{obs} values by nonlinear least-squares fitting of the data using OriginPro, version 8.0 (Northampton, MA, USA) (23), and k_2/K was determined from equation 5.

$$A = v_f \times t + (v_0 - v_f) \times (1 - e^{-k_{obs}\,t})/k_{obs} + A_0 \quad (4)$$

$$k_{obs} = k_{-2} + (k_2/K) \times [I]/(1 + [S]/K_m\,\mathrm{NCF}) \quad (5)$$

For equation 4, A is absorbance at λ_{482}, v_f is final velocity, t is time, v_0 is initial velocity, and A_0 is initial absorbance at λ_{482}. For equation 5, [I] is the concentration of AVI, [S] is the concentration of NCF, and k_{-2} is the recyclization rate constant. The data were plotted as k_{obs} versus [I], and the k_2/K value was obtained by correcting the value for the slope of the line for the concentration and affinity of NCF (equation 6):

$$k_2/K_{corrected} = k_2/K_{obs} \times ([S]/K_m\,\mathrm{NCF} + 1) \quad (6)$$

Using an Agilent 8453 diode array spectrophotometer, the k_{off} value was determined by incubating the PER-2 β-lactamase with an AVI concentration that resulted in complete inhibition. PER-2 (1 μM) was preincubated with 120 μM AVI (7.5× K_{iapp}). Samples were serially diluted (1:1,000), and hydrolysis of 100 μM NCF was measured. Progress curves were fit to a single exponential decay equation.

ESI-MS. To discern the nature of the intermediates of inactivation by AVI in the reaction pathway with the PER-2 β-lactamase, electrospray ionization mass spectrometry (ESI-MS) was performed using a Waters SynaptG2-Si quadrupole time of flight (QTOF) mass spectrometer equipped with a Waters Acquity H class Ultra Performance liquid chromatograph (UPLC) on an Acquity UPLC BEH C_{18} column (1.7-μm particle size; 2.1 by 100 mm), using standards and calibrations as previously described (28). For the experiments, the PER-2 β-lactamase was incubated with AVI for set times (i.e., 5 min and 24 h) at room temperature in 10 mM PBS, pH 7.4. The ratio of the inhibitor I (AVI) to PER-2 (or enzyme, E) was 1:1; the enzyme concentration was 20 μM. Reactions were terminated by the addition of 0.1% acetonitrile and 0.1% formic acid.

Molecular modeling of β-lactamases with AVI. Using Discovery Studio, version 3.1 (DS 3.1), molecular modeling software (Accelrys, Inc., San Diego, CA), PER-2 (PDB accession number 4D2O), CTX-M-15 (PDB 4HBT), and OXA-48 (PDB 4S2P) apo enzymes were compared (5, 15, 19). Also Michaelis complexes of PER-2 and CTX-M-15 with AVI were generated using the CDOCKER module (29). The AVI:enzyme complexes were solvated, the catalytic waters were added, and the models were minimized using a conjugate gradient with the CHARMm force field package and Shake algorithm constraints (root mean square [RMS] = 0.07 kcal/mole per Å). A standard dynamic cascade protocol was used to equilibrate the complexes for 200 ps.

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