5-4-2014

Reclaiming The Efficacy of β-Lactam–β-Lactamase Inhibitor Combinations: Avibactam Restores The Susceptibility of CMY-2-Producing Escherichia Coli to Ceftazidime

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Recommended Citation

Papp-Wallace, Krisztina M.; Winkler, Marisa L.; Gatta, Julian A.; Taracila, Magdalena A.; Chilakala, Sujatha; Xu, Yan; Johnson, J. Kristie; and Bonomo, Robert A., "Reclaiming The Efficacy of β-Lactam–β-Lactamase Inhibitor Combinations: Avibactam Restores The Susceptibility of CMY-2-Producing Escherichia Coli to Ceftazidime" (2014). *Chemistry Faculty Publications*. 210.
https://engagedscholarship.csuohio.edu/scichem_facpub/210

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Reclaiming the Efficacy of β-Lactam–β-Lactamase Inhibitor Combinations: Avibactam Restores the Susceptibility of CMY-2-Producing *Escherichia coli* to Ceftazidime

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CMY-2 is a plasmid-encoded Ambler class C cephalosporinase that is widely disseminated in *Enterobacteriaceae* and is responsible for expanded-spectrum cephalosporin resistance. As a result of resistance to both ceftazidime and β-lactamase inhibitors in strains carrying bla_{CMY}, novel β-lactam–β-lactamase inhibitor combinations are sought to combat this significant threat to β-lactam therapy. Avibactam is a bridged diazabicyclo [3.2.1]octanone non-β-lactam β-lactamase inhibitor in clinical development that reversibly inactivates serine β-lactamases. To define the spectrum of activity of ceftazidime-avibactam, we tested the susceptibilities of *Escherichia coli* clinical isolates that carry bla_{CMY-2} or bla_{CMY-69} and investigated the inactivation kinetics of CMY-2. Our analysis showed that CMY-2-containing clinical isolates of *E. coli* were highly susceptible to ceftazidime-avibactam (MIC_{90}, ≤0.5 mg/liter); in comparison, ceftazidime had a MIC_{90} of >128 mg/liter. More importantly, avibactam was an extremely potent inhibitor of CMY-2 β-lactamase, as demonstrated by a second-order onset of acylation rate constant (k_2/K) of $(4.9 \pm 0.5) \times 10^4$ M^{-1} s^{-1} and the off-rate constant (k_{off}) of $(3.7 \pm 0.4) \times 10^{-4}$ s^{-1}. Analysis of the reaction of avibactam with CMY-2 using mass spectrometry to capture reaction intermediates revealed that the CMY-2–avibactam acyl-enzyme complex was stable for as long as 24 h. Molecular modeling studies raise the hypothesis that a series of successive hydrogen-bonding interactions occur as avibactam proceeds through the reaction coordinate with CMY-2 (e.g., T316, G317, S318, T319, S343, N346, and R349). Our findings support the microbiological and biochemical efficacy of ceftazidime-avibactam against *E. coli* containing plasmid-borne CMY-2 and CMY-69.

Biochemical studies of avibactam have shown that this inhibitor inactivates β-lactamases with second-order acylation rate constants (k_2/K) ranging from 1,400 to 160,000 M^{-1} s^{-1} (1–6). The highest acylation rates were those for class A enzymes, while the lowest were those for class D β-lactamases. Dissociation rate constants (k_{off}) ranged from $(1.9 \pm 0.6) \times 10^{-3}$ to $(1.2 \pm 0.4) \times 10^{-5}$ s^{-1} and were lowest for class D β-lactamases (6). Most importantly, avibactam combined with either ceftazidime, ceftaroline (the active metabolite of ceftaroline fosamil), or ceftaroline-fosamil was effective in murine models of infection due to highly resistant extended-spectrum β-lactamase (ESBL)-producing, non-ESBL-producing, and *Klebsiella pneumoniae* carbapenemase (KPC)-producing *Enterobacteriaceae* and *Pseudomonas aeruginosa* isolates (7–10).

CMY-2 β-lactamase is the most prevalent and widely disseminated plasmid-borne cephalosporinase (11, 12). As such, *Escherichia coli* strains possessing bla_{CMY} pose one of the most important challenges to the efficacy of β-lactam therapy in both community- and hospital-acquired infections. Inhibition studies with CMY-2 and tazobactam, for example, were important in helping to understand the path to the inhibition of AmpC enzymes (13, 14).

To date, two studies have described the potent activities of ceftazidime-avibactam and ceftaroline (the active metabolite of ceftaroline fosamil)-avibactam against strains producing bla_{CMY-2}; however the biochemical basis of the activity of avibactam against CMY-2 has not yet been defined (15, 16). In this work, a genetically diverse population of *E. coli* clinical isolates that carry either bla_{CMY-2} or bla_{CMY-69} was tested for susceptibility to ceftazidime-avibactam. In addition, we extend previous analyses of CMY-2 by describing, for the first time, the details of the inhibition of purified CMY-2 by avibactam and modeling avibactam within the active site of CMY-2 in order to gain insight into the efficacy of this novel inhibitor against this important clinical resistance determinant.

MATERIALS AND METHODS

Bacterial strains, plasmids, and mutagenesis. The ceftazidime-resistant *E. coli* clinical isolates used in this study were obtained from the University of Maryland as part of a perianal surveillance study. The goal of that study was to look at admission colonization versus acquisition of plasmid-mediated AmpC enzymes in the medical intensive care unit (MICU) and the surgical intensive care unit (SICU) over time.

The cloning of bla_{CMY-2} into the pBC SK(−) phagemid vector (Stratagene, La Jolla, CA) for antimicrobial susceptibility testing (AST) and protein expression in *E. coli* DH10B cells (Invitrogen Corp., Carlsbad, CA) was described previously (14). The QuikChange XL site-directed

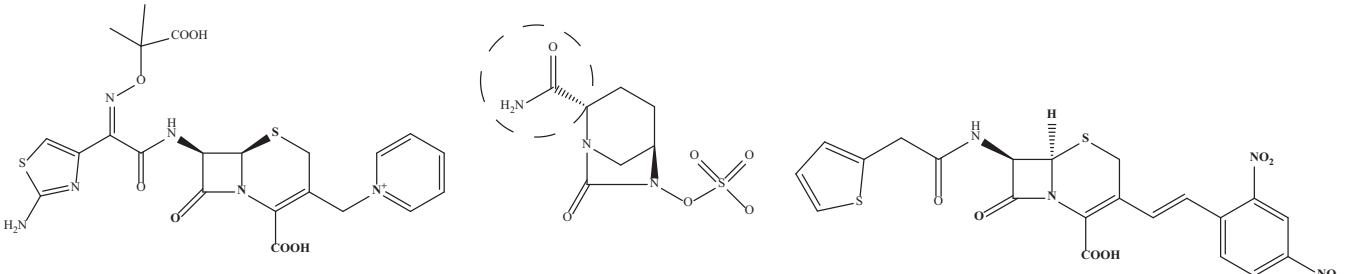

FIG 1 Compounds used in this study. A dashed circle represents the carboxamide of avibactam.

mutagenesis kit (Agilent Technologies, Santa Clara, CA) was used to perform site-directed mutagenesis of $bla_{\text{CMY-2}}$ to produce $bla_{\text{CMY-69}}$ according to the manufacturer's protocol.

PFGE. Pulsed-field gel electrophoresis (PFGE) was performed as reported previously with the exception of the electrophoresis parameters listed here (17). Briefly, genomic bacterial DNA was digested with XbaI for 4 h at 37°C. Electrophoresis was performed on the CHEF-DR II system using a 1% agarose gel at 200 V for 22 h, with an initial switch time of 2.2 s and a final switch time of 54.2 s. Gels were stained with ethidium bromide and were then analyzed using GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium). A dendrogram that compared all isolates was constructed using GelCompar II software with the Dice coefficient and the unweighted-pair group method with arithmetic means and with a position tolerance of 1. The banding patterns of isolates required a >2-band difference for the isolate to be considered a unique strain (18).

Determination of bla_{CMY} background in strains. Plasmid preparations from plasmids conjugated into *E. coli* J53 were used to amplify bla_{CMY} by using the protocol published previously by Hanson et al. (19). The amplified genes were sequenced using the following primers: CMY2-F2 (ACGCTAACTCCAGCATTGGT) and CMY2-R2 (CAAACAG ACCAATGCTGGAG). Sequencing data were assembled with Sequencher, version 5.0 (Gene Codes, Ann Arbor, MI).

Identification of bla_{TEM} and bla_{SHV} in strains. Total bacterial DNA was used to amplify bla_{TEM} and bla_{SHV} according to previously published methods (20, 21).

AST. MICs for various bacterial isolates were determined by the Mueller-Hinton agar dilution method according to the Clinical and Laboratory Standards Institute guidelines (22). The MICs were measured using a Steers replicator that delivered 10 μl of a diluted overnight culture containing 10^4 CFU. Avibactam (AstraZeneca, Waltham, MA) was tested at 4 mg/liter in combination with increasing concentrations of ceftazidime (Sigma-Aldrich). The structures of the compounds used in this study are shown in Fig. 1.

Protein purification. The CMY-2 β-lactamase was purified as described previously (14). Briefly, cultures were grown at 37°C in Super Optimal Broth (SOB). The cells were then pelleted and were subjected to stringent periplasmic fractionation. For periplasmic fractionation, cells were incubated with lysozyme, Benzonase nuclease, and magnesium sulfate for 25 min, and with EDTA for an additional 5 min, and were then centrifuged to pellet cellular debris. The enzyme was purified by preparative isoelectric focusing using the crude extracts, and then fast-protein liquid chromatography purification was performed using a HiLoad 16/60 Superdex 75 gel filtration column. The purities of the proteins were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were determined to be >95%. Protein concentrations were determined by a spectrophotometric assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard according to the manufacturer's instructions (14).

Steady-state kinetic analysis. Kinetic parameters were determined using an Agilent (Santa Clara, CA) 8453 diode array spectrophotometer. All reactions were conducted in 10 mM phosphate-buffered saline (PBS) at pH 7.4 at room temperature.

The K_m and $V_{\max}$ for CMY-2 and nitrocefin (NCF) were determined by maintaining enzyme concentrations at 3 nM and using NCF in excess molar concentration to establish pseudo-first-order kinetics. The subsequent data were fit to the Henri-Michaelis-Menten equation using EnzFitter software (Biosoft Corporation, Ferguson, MO).

For the purposes of this study, the interactions between CMY-2 and avibactam are represented according to the following scheme, which is based on previous work with TEM-1 and avibactam (4, 23):

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{:}I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E - I$$

where E is the enzyme, I is the inhibitor, k_1 is the association rate constant, k_{-1} is the dissociation rate constant, k_2 is the acylation rate constant, and k_{-2} is the recyclization rate constant.

The determination of the apparent K_i ($K_{i\ \text{app}}$) has been described previously (24). $K_{i\ \text{app}}$ was determined for CMY-2 by using a direct competition assay under steady-state conditions. CMY-2 was maintained at 3 nM while the avibactam concentration was varied. NCF was used as the reporter substrate at a fixed concentration of 100 μM. CMY-2 β-lactamase, avibactam, and NCF were mixed manually, and the reaction velocity for the first 5 s of the reaction was monitored. Data were linearized using a Dixon plot of inverse initial steady-state velocities ($1/V_0$) versus inhibitor concentration [I]. $K_{i\ \text{app}}$ was determined by dividing the value for the y intercept by the slope of the line (25). The data were corrected to account for NCF ($K_m = 11 \pm 1$ μM) for the β-lactamase using equation 1:

$$K_{i\ \text{app}}\left(\text{corrected}\right) = K_{i\ \text{app}}\left(\text{observed}\right) / \left\{1 + \left(\left[S\right] / \left[K_m \ \ \text{NCF}\right]\right)\right\} \tag{1}$$

where [S] is the concentration of substrate or NCF.

To obtain the onset of acylation k_2/K, progress curves were obtained by mixing CMY-2 at 5 nM with increasing concentrations of avibactam using NCF at 100 μM as a reporter substrate (4). Progress curves were fit to equation 2 to obtain the observed rate constant for inactivation (k_{obs}).

$$y = V_f \cdot x + \left(V_0 - V_f\right) \cdot \left[1 - \exp\left(-k_{\text{obs}} \cdot X\right)\right] / k_{\text{obs}} + A_0 \tag{2}$$

Here, V_f is the final velocity, V_0 is the initial velocity, and A_0 is the initial absorbance at a λ of 482 nm. The data were plotted as k_{obs} versus [avibactam]. The k_2/K value was obtained by correcting the value obtained for the slope of the line (k_2/K observed) for the use of NCF (K_m NCF = 11 ± 1 μM) as an indicator substrate (equation 3).

$$k_2/K\ (\text{corrected}) = k_2/K\ (\text{observed}) \cdot \left\{\left(\left[S\right]/K_m \ \ \text{NCF}\right) + 1\right\} \tag{3}$$

Partition ratios ($k_{\text{cat}}/k_{\text{inact}}$ [where k_{inact} is the rate constant of enzyme inactivation]) at 24 h for β-lactamases with avibactam were obtained by incubating CMY-2 with increasing concentrations of avibactam at room temperature in 10 mM PBS, pH 7.4. The ratio of inhibitor to enzyme ($I{:}E$) necessary to inhibit the hydrolysis of NCF by >99% was determined.

The k_{off} of avibactam for CMY-2 was determined using a previously published method (4). In these experiments, 3.9 μM CMY-2 was incubated with 19.5 μM avibactam for 5 min at room temperature; the mixture was serially diluted to a final CMY-2 concentration of 390 pM; and 100 μM NCF was added. Progress curves measuring NCF hydrolysis were collected for 1 h, and the data were fit to equation 2 to obtain k_{off}. CMY-2 alone and avibactam alone were used as controls.

ESI-MS. To discern the nature of the intermediates of inactivation by avibactam in the reaction pathway with the CMY-2 β-lactamase, electrospray ionization (ESI) mass spectrometry (MS) was performed on an Applied Biosystems (Foster City, CA) QStar Elite quadrupole time-of-flight mass spectrometer equipped with a TurboIonSpray source. The CMY-2 β-lactamase was incubated with avibactam for a set time (i.e., 5 min or 24 h) at room temperature in 10 mM PBS, pH 7.4. The ratios of the inhibitor (*I*) (avibactam) to the enzyme (*E*) (CMY-2) were approximately 1:1. The reactions were terminated by the addition of 0.2% formic acid. All samples were desalted and were concentrated by using a C_{18} ZipTip pipette tip (Millipore, Billerica, Massachusetts) according to the manufacturer's protocol. Eluted protein samples were diluted with 50% acetonitrile and 0.2% formic acid to a concentration of 10 μM and were infused at a rate of 0.5 μl per min, and data were collected for 2 min. The source temperature was set at 300°C with an ion spray voltage of 5,500 V and with declustering potential 1 (DP1) at 70 V and DP2 at 10 V. Spectra were deconvoluted using the Applied Biosystems Analyst program.

Molecular modeling. The crystal structure of CMY-2 (PDB identification [ID] 1ZC2) was used to construct Michaelis-Menten and acyl-enzyme complexes with avibactam as described previously using Discovery Studio, version 3.1 (DS 3.1) (Accelrys, Inc., San Diego, CA), molecular modeling software (26). Avibactam was constructed using Fragment Builder tools and was minimized using a Standard Dynamics Cascade protocol of DS 3.1. The compound was automatically docked into the active site of CMY-2 by using the CDOCKER module of DS 3.1. This protocol uses a CHARMm-based molecular dynamics scheme to dock ligands into a receptor-binding site.

Molecules were automatically typed by the CHARMm force field using the Momany-Rone partial charge estimation method (27). This method uses an algorithm that attempts to match atoms in the molecule with those defined by the force field atom templates. When a match is found, the force field types and partial charges from the template are assigned to those atoms in the molecule. CHARMm was chosen because it is a general-purpose all-atom force field with wide coverage for proteins, nucleic acids, and general organic molecules.

The best conformations were automatically aligned to polar and apolar active-site "hot spots," and the best-scoring poses were reported. At this step, the hydrogen atoms were not maintained. To further optimize the docked poses (i.e., to add hydrogens and prevent clashes between the receptor and ligand), a CHARMm minimization step was used. Here, the SMART (simultaneous multiplicative algebraic reconstruction technique) minimization algorithm was employed (i.e., 1,000 steps of steepest descent with a root mean square [RMS] gradient tolerance of 3 Å, followed by conjugate gradient minimization, with an RMS deviation [RMSD] minimization gradient of 0.001 Å). For the final minimization of the avibactam conformations docked into the active site of CMY-2, an RMSD cutoff of 1 Å was chosen.

The resulting conformations of CMY-2–avibactam complexes were analyzed; the most favorable positioning of avibactam was chosen (i.e., the C-7 carbonyl of avibactam oriented toward the amide backbones of Ser64 and Ser318); and the complexes between the enzyme and inhibitor were created, as described previously (28). To check the stability of the complexes, 6-ps molecular dynamics simulations (MDS) were conducted for the CMY-2–avibactam Michaelis and acyl-enzyme complexes (28). During the heating/cooling, equilibration, and production stages of MDS, a temperature of 300 K and a constant pressure were maintained. The long-range electrostatic interactions were treated with the Particle Mesh Ewald method and explicit solvation with Periodic Boundary Conditions. The MDS and the production step of MDS for CMY-2–avibactam complexes were run without any constraints.

RESULTS AND DISCUSSION

CMY-producing *E. coli* strains are genetically diverse. We detected bla_{CMY-2} in 27 of the 28 isolates tested; 1 isolate possessed bla_{CMY-69}. In addition, five isolates carried bla_{TEM}, and two strains possessed bla_{SHV}. bla_{TEM} and bla_{SHV} PCR amplicons were not subjected to DNA sequencing. PFGE analysis of the 28 clinical *E. coli* isolates carrying bla_{CMY-2} or bla_{CMY-69} revealed that these strains made up a genetically heterogeneous population (Table 1).

Avibactam combined with ceftazidime restores the susceptibility of *E. coli* carrying bla_{CMY} to ceftazidime. AST was conducted on the *E. coli* clinical strains carrying bla_{CMY-2} or bla_{CMY-69}, and the results are summarized in Table 1. Ceftazidime MICs ranged from 16 to >128 mg/liter. The ceftazidime-avibactam combination lowered MICs to 0.5 mg/liter against all isolates tested in this collection of diverse *E. coli* strains carrying bla_{CMY-2}. The ceftazidime-avibactam combination displayed a higher MIC (i.e., 2 mg/liter) against a single isolate (strain 9592) possessing bla_{CMY-69} than against the bla_{CMY-2}-producing isolates.

Compared to CMY-2, the CMY-69 β-lactamase contained a single amino acid substitution, A295P. To determine the contribution of bla_{CMY-69} to the ceftazidime and ceftazidime-avibactam MIC results, bla_{CMY-2} was altered to produce bla_{CMY-69}, which was expressed from the pBC SK(−) phagemid in an *E. coli* DH10B background. The MICs of ceftazidime and ceftazidime-avibactam were determined by the agar dilution method, and difference in ceftazidime-avibactam MICs was not observed (Table 1). Thus, the expression of bla_{CMY-69} was not the determining factor for the elevated MIC against isolate 9592; porin loss, the expression of another β-lactamase (the strain possesses TEM), and/or the expression of an efflux pump may potentially play a role. A previous study showed that porin loss in *Enterobacter cloacae* can contribute to elevated ceftaroline-avibactam MICs (29).

CMY-2 is inactivated by avibactam and maintains a stable acyl-enzyme complex. The inhibitory ability of avibactam compared to other β-lactamase inhibitors against CMY-2 was determined and is presented in Tables 2 and 3. Progress curves measuring NCF hydrolysis were obtained for CMY-2 by using increasing concentrations of avibactam (range, 0.5 to 10 μM) as a competitor (Fig. 2A). Progress curves were fit using equation 2 to obtain k_{obs} values. k_{obs} values were plotted against avibactam concentrations. The results indicated fast acylation and weak encounter complex binding for avibactam and CMY-2 (Fig. 2B) (4). The corresponding k_2/K value obtained for CMY-2 revealed that CMY-2 was inactivated with a second-order rate constant of $(4.9 \pm 0.5) \times 10^4$ M^{-1} s^{-1}. The k_{off} value of $(3.7 \pm 0.4) \times 10^{-4}$ s^{-1} suggested that avibactam deacylated from CMY-2 slowly (Fig. 2C).

Analysis using mass spectrometry showed that the CMY-2–avibactam acyl-enzyme complex was stable for as long as 24 h (Fig. 2D). Avibactam is a reversible inhibitor; thus, even if avibactam deacylates from the CMY-2 β-lactamase during the 24 h, avibactam remains in an active form. Thus, given the rapid acylation rate of avibactam for CMY-2, free CMY-2 would not be observed using mass spectrometry. In addition to the expected increase in mass as a result of avibactam (+264 ± 5 atomic mass units [amu]), the

TABLE 1 MICs of ceftazidime and ceftazidime-avibactam for the various *E. coli* clinical isolates containing bla_{CMY}

E. coli strain	CMY	Presence of TEM or SHV	PFGE result[a]	MIC (mg/liter)[b]	
				Ceftazidime	Ceftazidime-avibactam
DH10B		No	ND	0.12	<0.06
DH10B/pBC SK(−) bla_{CMY-2}	CMY-2	No	ND	32	0.12
DH10B/pBC SK(−) bla_{CMY-69}	CMY-69	No	ND	64	0.12
134	CMY-2	TEM	1	32	<0.06
660	CMY-2	No	2	64	<0.06
9592	CMY-69	TEM	3	>128	2
9292	CMY-2	No	4	128	0.12
9310	CMY-2	No	5	32	<0.06
9461	CMY-2	No	6	16	<0.06
9614	CMY-2	TEM	7a	32	<0.06
9790	CMY-2	No	8	64	0.12
10689	CMY-2	No	9	32	<0.06
10924	CMY-2	No	10	>128	0.5
10927	CMY-2	TEM	11a	64	0.12
11356	CMY-2	No	12	64	0.12
11521	CMY-2	No	3	64	<0.06
11584	CMY-2	No	11b	32	<0.06
11813	CMY-2	No	13	32	<0.06
8793	CMY-2	SHV	7b	>128	0.5
2728	CMY-2	SHV	14	16	<0.06
2758	CMY-2	No	15	16	<0.06
3113	CMY-2	No	16	16	<0.06
3288	CMY-2	No	17	32	<0.06
3464	CMY-2	No	18	32	0.12
4139	CMY-2	No	19	>128	0.25
5275	CMY-2	No	20	16	<0.06
5336	CMY-2	No	21	32	0.12
6968	CMY-2	No	22	16	<0.06
7673	CMY-2	No	23	32	<0.06
8117	CMY-2	TEM	24	32	<0.06
8218	CMY-2	NO	25	16	<0.06

[a] Isolates with the same number and no letter are identical. Isolates with the same number and "a" or "b" are closely related. ND, not determined (control strains).
[b] Avibactam was maintained at a constant concentration of 4 mg/liter in the ceftazidime-avibactam combinations. MICs were measured in triplicate, and the mode is presented.

addition of another +184 ± 5 amu was observed in the avibactam incubations, and the fixed proportion of this mass peak from 5 min to 24 h suggests that it is a mass spectrometry ionization artifact (6, 30).

Insights and hypotheses about the potent inhibition profile of avibactam against CMY-2. To obtain a broader perspective on the inhibition of class C β-lactamases by avibactam, a molecular model using the crystal structure of the CMY-2 apoenzyme with avibactam docked into the active site was compared to the crystal structure of the *P. aeruginosa* PAO1 AmpC enzyme PDC-1 with avibactam (31). We chose PDC-1 because it is the only class C β-lactamase that was characterized kinetically and that possessed a solved avibactam acyl-enzyme structure (PDB ID 4HEF) (5, 6).

TABLE 2 Kinetic parameters for CMY-2 β-lactamase

Parameter	CMY-2
NCF K_m (μM)	11 ± 1
K_i (μM)	
Clavulanic acid	4,365 ± 471[a]
Tazobactam	50 ± 10[a]
Sulbactam	101 ± 8[a]

[a] Data from reference 14.

Intact avibactam was docked into the active site of the apo-CMY-2 crystal structure (PDB ID 1ZC2) (Fig. 3A). The C-7 carbonyl of avibactam was positioned within the oxyanion hole formed by residues Ser64 (2.9 Å) and Ser318 (3.0 Å). We recognize that the general base involved in the acylation of avibactam is debated for class C β-lactamases (32); Tyr150 and Lys67 are hypothesized to be involved in the deprotonation of Ser64 (33–40). The molecular representation generated here revealed that both Tyr150 (3.0 Å) and Lys67 (2.8 Å) can form hydrogen-bonding interactions with the hydroxyl side chain of the nucleophilic residue Ser64, suggesting that either residue may be involved in the acylation mechanism of avibactam. In addition, a water molecule observed within hydrogen-bonding distance of Tyr150 (3.0 Å) and Ser64 (2.8 Å) may play a role in proton transfer for acylation.

Our simulation next revealed that the oxygen of the carboxamide of avibactam was within hydrogen-bonding distance of Lys67 (2.6 Å). As anticipated, a dynamic hydrogen-bonding network consisting of residues Thr316, Gly317, Ser318, Thr319, Ser343, Asn346, and Arg349 (with Ser318 [2.7 Å], Thr319 [3.0 Å], and Ser343 [3.0 Å] interacting directly with avibactam) was formed

TABLE 3 Kinetic parameters with avibactam

Parameter	β-Lactamase		
	CMY-2	P99	PDC-1
$K_{i\ app}$ (μM) avibactam	26 ± 3	Not available	Not available
k_{cat}/k_{inact}	1	Not available	Not available
k_2/K (M^{-1} s^{-1})	$(4.9 \pm 0.5) \times 10^4$	$(5.1 \pm 0.1) \times 10^{3a}$	$(2.9 \pm 0.1) \times 10^{3a}$
k_{off} (s^{-1})	$(3.7 \pm 0.4) \times 10^{-4}$	$(3.8 \pm 0.2) \times 10^{-5a}$	$(1.9 \pm 0.60) \times 10^{-3a}$
K_d (nM)[b]	7.5 ±0.7	7[a]	660[a]

[a] Data from reference 6.
[b] K_d, dissociation constant.

and stabilized the avibactam complex and the negatively charged sulfate group of avibactam. This region of the active site is the proposed β-lactam C-3/C-4 carboxylate binding site in class C β-lactamases.

Upon the formation of the carbamate bond between avibactam and CMY-2 Ser64, the C-7 carbonyl was still positioned within the oxyanion hole, between Ser64 (2.8 Å) and Ser318 (2.8 Å) (Fig. 3B). However, Lys67 moved >4.0 Å away from Ser64, while Tyr150 (3.0 Å) remained with hydrogen-bonding distance. In this part of the simulation, the water molecule shifted more than 3 Å and moved away from Ser64 toward Lys315, which also moved 3 Å away from the active site. Interestingly, the carboxamide of

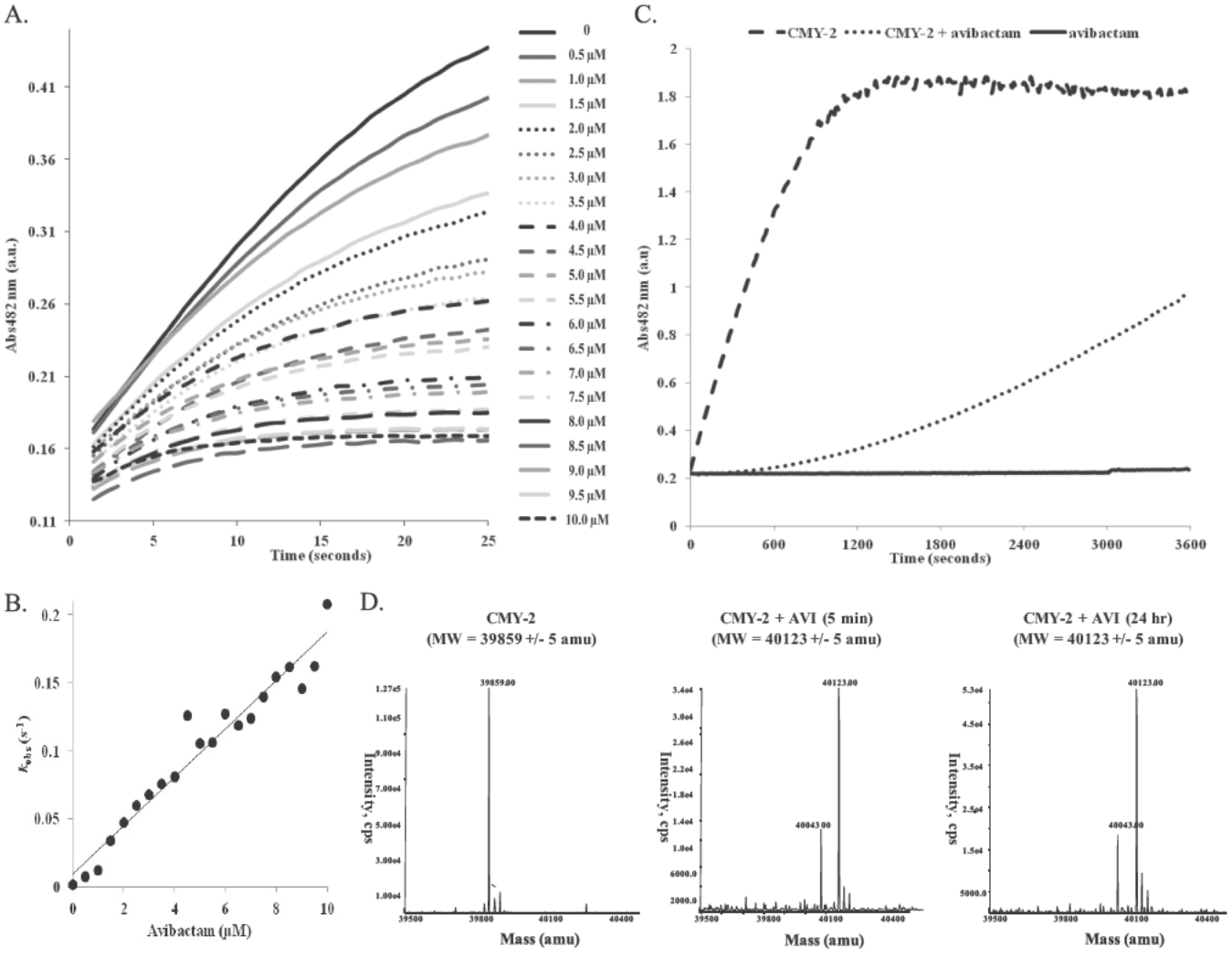

FIG 2 (A) Progress curves obtained by using increasing concentrations of avibactam (0.5 μM to 10.0 μM) against fixed concentrations of CMY-2 (5 nM) and the reporter substrate NCF (100 μM). (B) The k_{obs} values obtained by fitting the progress curves in panel A to equation 2 were plotted against the avibactam concentration. (C) Progress curves for CMY-2–avibactam and controls (i.e., CMY-2 alone and avibactam alone) used to determine the off rate. The CMY-2–avibactam curve was fit to equation 2 in order to determine k_{off}. (D) ESI-MS of CMY-2 alone (left) and of CMY-2–avibactam after a 5-min (center) or a 24-h (right) incubation.

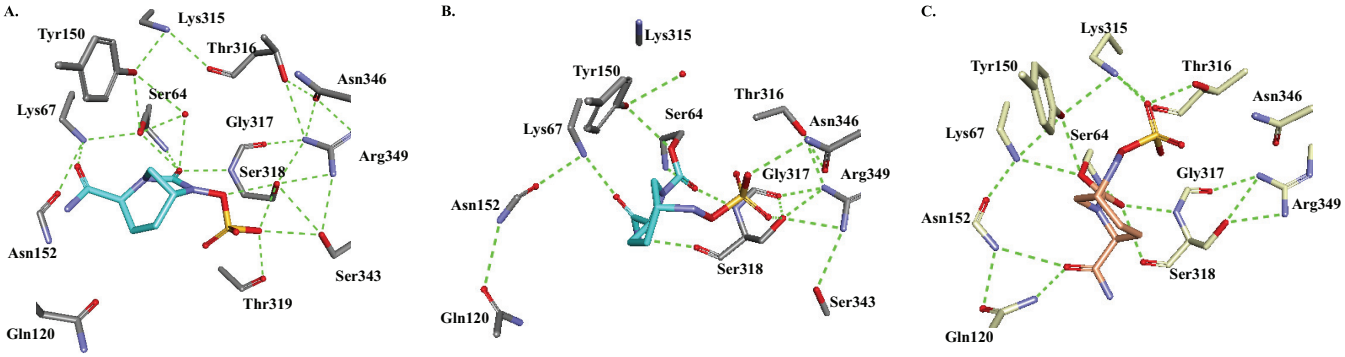

FIG 3 (A and B) Molecular models of the Michaelis complex (A) and the acyl-enzyme complex (B) of CMY-2 (gray) and avibactam (cyan). As determined by MDS, a series of successive hydrogen-bonding interactions occur as avibactam proceeds through the reaction coordinate (i.e., T316, G317, S318, T319, S343, N346, and R349). (C) "Snapshot" of the crystal structure of PDC-1 (yellow) with avibactam (orange). In all panels, dashed green lines indicate potential hydrogen-bonding interactions.

avibactam was still within hydrogen-bonding distance of Lys67 (2.5 Å) but now also was able to form hydrogen-bonding interactions with Ser318 (2.8 Å). In addition, the sulfate rotated 90° toward Asn346 and was within hydrogen-bonding distance of Ser318 (3.0 Å) and Asn346 (2.9 Å). In this pose, the complex hydrogen-bonding network consisting of residues Thr316, Gly317, Ser318, Thr319, Ser343, Asn346, and Arg349 was again present.

Much as with acylation, the mechanism of deacylation of class C β-lactamases is uncertain; however, Tyr150 is believed to be the general base, with Lys67 involved in proton shuttling (32, 37, 41). Continuing our analysis, we observed a water molecule within hydrogen-bonding distance of Tyr150. Thus, potential for hydrolytic deacylation exists. However, the mass spectrometry studies that we performed revealed that avibactam is not hydrolyzed effectively by CMY-2. The identity of the general base that removes a proton from the secondary amine of avibactam in order for recyclization (4–6) to occur is unclear, because no such residues are within 3 Å of this proton. Thus, since neither hydrolysis nor recyclization are likely events, the low k_{off} value of CMY-2 is supported by our model.

Last, we compared the molecular representation of the CMY–avibactam acyl-enzyme complex to the crystal structure of PDC-1 with avibactam (PDB ID 4HEF) (Fig. 3C). The observable differences between the structure and the model are that (i) the carboxamide of avibactam in complex with PDC-1 was within hydrogen-bonding distance of Gln120 (3.0 Å) and Asn152 (3.0 Å); (ii) the sulfate was more buried in the active site of PDC-1 than in that of CMY-2 in our model as a result of hydrogen-bonding interactions with the side chains of Lys315 (3.0 Å) and Thr316 (2.6 Å); and (iii) there were more water molecules within the active site of PDC-1 than in that of CMY-2 (5).

Conclusion. Previous studies have reported that ceftazidime-avibactam was effective against strains with complex β-lactamase backgrounds (1, 2, 15, 16, 26, 30, 42–49). In our analysis, ceftazidime-avibactam was a potent antibiotic combination against a diverse collection of clinically derived *E. coli* strains bearing CMY β-lactamase. Specifically, when avibactam was combined with ceftazidime, ceftazidime MICs against *E. coli* strains producing bla_{CMY-2} and bla_{CMY-69} were lowered. In addition, purified CMY-2 was rapidly inactivated by avibactam; deacylation of the acyl-enzyme complex was slow; and the complex was stable for as long as 24 h. These observations were supported by molecular modeling of CMY-2 with avibactam, since the acyl-enzyme complex appeared stable; however, the questions of why deacylation hydrolysis would not occur and how recyclization of avibactam would take place remain to be determined.

Overall, our data show why avibactam will be a significant addition to the antibiotic armamentarium against *E. coli* strains producing bla_{CMY-2} and bla_{CMY-69}. Despite the diversity of these strains, we note that the size of this bacterial collection limits the results obtained. However, the clinical implications of these findings are significant, since the dissemination of plasmidic AmpC enzymes in *E. coli* is a major clinical threat. These studies provide important findings that support the future development of novel β-lactamase inhibitors.

ACKNOWLEDGMENTS

This work was supported by a research grant from AstraZeneca (to K.M.P.-W. and R.A.B.). The study was also supported in part by funds and/or facilities provided by the Cleveland Department of Veterans Affairs, the Department of Veterans Affairs Career Development Program (K.M.P.-W.), Department of Veterans Affairs Merit Review Program award 1I01BX001974 (R.A.B.), and the Veterans Integrated Service Network 10 Geriatric Research, Education, and Clinical Center (VISN 10 GRECC) (R.A.B.). Research reported in this publication was also supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under award numbers R01AI100560 and R01AI063517 to R.A.B. M.L.W. has been supported by a Medical Scientist Training Program training grant, Case Western Reserve University-T32 GM07250. J.K.J. is supported by Career Development award 1K12RR023250 and by funding from BioFire and Nanosphere.

The content of this article is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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