



# Relebactam Is a Potent Inhibitor of the KPC-2 $\beta$ -Lactamase and Restores Imipenem Susceptibility in KPC-Producing *Enterobacteriaceae*

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**ABSTRACT** The imipenem-relebactam combination is in development as a potential treatment regimen for infections caused by *Enterobacteriaceae* possessing complex  $\beta$ -lactamase backgrounds. Relebactam is a  $\beta$ -lactamase inhibitor that possesses the diazabicyclooctane core, as in avibactam; however, the R1 side chain of relebactam also includes a piperidine ring, whereas that of avibactam is a carboxamide. Here, we investigated the inactivation of the *Klebsiella pneumoniae* carbapenemase KPC-2, the most widespread class A carbapenemase, by relebactam and performed susceptibility testing with imipenem-relebactam using KPC-producing clinical isolates of *Enterobacteriaceae*. MIC measurements using agar dilution methods revealed that all 101 clinical isolates of KPC-producing *Enterobacteriaceae* (*K. pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Citrobacter koseri*, and *Escherichia coli*) were highly susceptible to imipenem-relebactam (MICs  $\leq$  2 mg/liter). Relebactam inhibited KPC-2 with a second-order onset of acylation rate constant ( $k_2/K$ ) value of 24,750 M<sup>-1</sup> s<sup>-1</sup> and demonstrated a slow off-rate constant ( $k_{off}$ ) of 0.0002 s<sup>-1</sup>. Biochemical analysis using time-based mass spectrometry to map intermediates revealed that the KPC-2–relebactam acyl-enzyme complex was stable for up to 24 h. Importantly, desulfation of relebactam was not observed using mass spectrometry. Desulfation and subsequent deacylation have been observed during the reaction of KPC-2 with avibactam. Upon molecular dynamics simulations of relebactam in the KPC-2 active site, we found that the positioning of active-site water molecules is less favorable for desulfation in the KPC-2 active site than it is in the KPC-2–avibactam complex. In the acyl complexes, the water molecules are within 2.5 to 3 Å of the avibactam sulfate; however, they are more than 5 to 6 Å from the relebactam sulfate. As a result, we propose that the KPC-2–relebactam acyl complex is more stable than the KPC-2–avibactam complex. The clinical implications of this difference are not currently known.

**KEYWORDS**  $\beta$ -lactams,  $\beta$ -lactamases, relebactam, carbapenemase,  $\beta$ -lactamase inhibitor

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The KPC-2  $\beta$ -lactamase is the most prevalent carbapenemase in carbapenem-resistant *Enterobacteriaceae* (CRE) in the United States and is disseminated worldwide. KPC-2's spectrum of activity includes all currently available  $\beta$ -lactams and clavulanic acid, sulbactam, and tazobactam (1). Fortunately, KPC-2 is found to be susceptible to inhibition by diazabicyclooctane (DBO)  $\beta$ -lactamase inhibitors (2, 3). Relebactam, formerly MK-7655, is a DBO that promises to contribute to the renaissance in antimicrobial chemotherapy (Fig. 1) (2). When combined with imipenem, relebactam is effective against *Enterobacteriaceae* with known KPC carbapenemases, AmpCs, and/or extended-spectrum  $\beta$ -lactamases (ESBLs) (2, 4, 5). Imipenem-relebactam also demonstrates potent activity against *Enterobacteriaceae* that express AmpCs or ESBLs and that have impermeability phenotypes (e.g., loss of porins) (5). Investigators showed that the imipenem-relebactam combination proved to be very effective in a hollow-fiber infection model due to a KPC-producing *Klebsiella pneumoniae* strain (4, 6). Furthermore, in mouse models of infection with imipenem-resistant *Klebsiella* strains, imipenem-relebactam performed favorably compared to imipenem alone (7). The goal of this investigation was to assess the efficacy of imipenem-relebactam against a panel of clinical isolates of KPC-producing *Enterobacteriaceae* and determine the inhibition parameters for purified KPC-2 with relebactam.

## RESULTS AND DISCUSSION

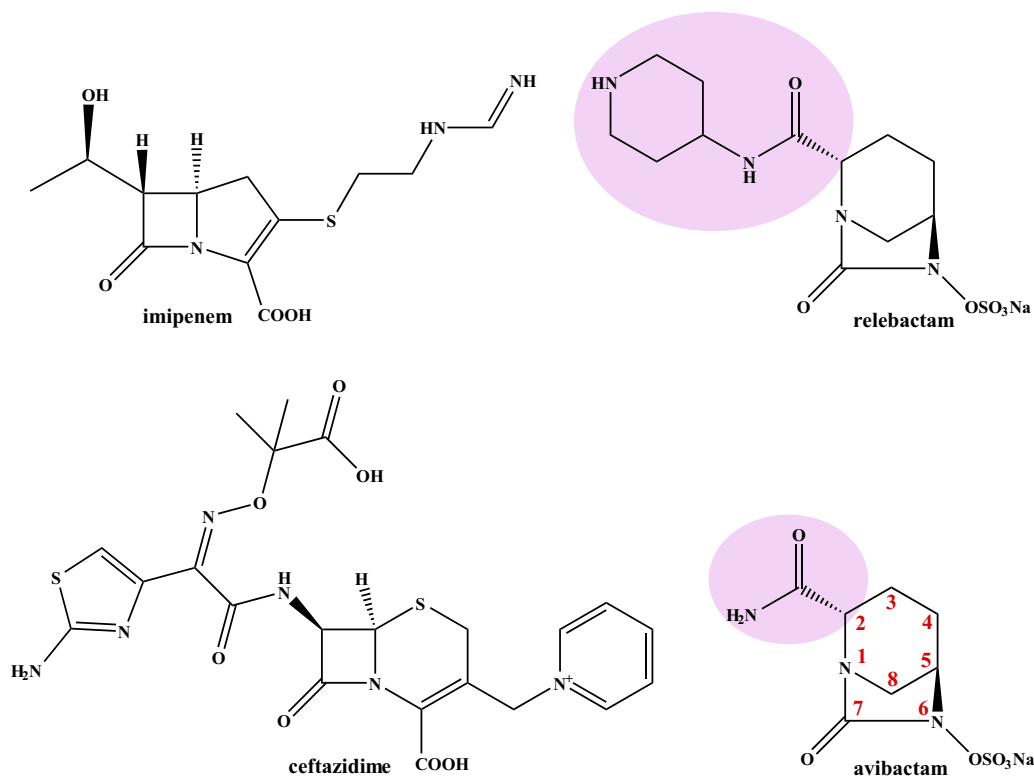
**Imipenem-relebactam demonstrates potent antimicrobial activity against KPC producers.** As the combination of imipenem-relebactam is being developed to target *Enterobacteriaceae* with  $bla_{KPC5}$ , we tested the activity of imipenem and imipenem-relebactam as well as ceftazidime and ceftazidime-avibactam for comparison against a select panel of 101 isolates of the *Enterobacteriaceae* expressing  $bla_{KPC5}$  (Table 1). All isolates tested were susceptible to both ceftazidime-avibactam and imipenem-relebactam (Table 2).

**Relebactam is an effective inhibitor of KPC-2.** The values of the apparent  $K_i$  ( $K_{i,app}$ ), the second-order onset of the acylation rate constant ( $k_2/K$ ), the off-rate constant ( $k_{off}$ ), and the  $k_{cat}/$ rate constant of enzyme inactivation ( $k_{inact}$ ) were determined for purified KPC-2 with relebactam using nitrocefirin as a reporter substrate. Previously for nitrocefirin, KPC-2 was found to possess a  $K_m$  of 8  $\mu$ M and a  $k_{cat}$  of 130  $s^{-1}$ , for a  $k_{cat}/K_m$  ratio of 16  $\mu$ M $^{-1}$   $s^{-1}$  (8). Comparatively, imipenem is a poorer substrate with a  $K_m$  of 21  $\mu$ M and a  $k_{cat}$  of 21  $s^{-1}$ , for a  $k_{cat}/K_m$  ratio of 1.0  $\mu$ M $^{-1}$   $s^{-1}$  (8). To determine the relative affinity of relebactam for KPC-2, a direct competition assay between 25  $\mu$ M nitrocefirin and increasing concentrations of relebactam measuring initial velocities for nitrocefirin hydrolysis was used. The  $K_{i,app}$  value of relebactam for KPC-2 was  $2.3 \pm 0.3$   $\mu$ M, which is similar to that of avibactam,  $1.0 \pm 0.1$   $\mu$ M (Fig. 2A and Table 3) (3, 9).

To determine the  $k_2/K$  or acylation rate of relebactam for KPC-2, progress curves were obtained for the hydrolysis of 50  $\mu$ M nitrocefirin in direct competition with increasing concentrations of relebactam (Fig. 2B). The values of the observed rate constant for inactivation ( $k_{obs}$ ) were determined and plotted versus the concentrations of inhibitor (Fig. 2C). The slope of the line corresponds to the  $k_2/K$  observed value, which was corrected for the use of nitrocefirin. The  $k_2/K$  value for relebactam inactivating KPC-2 was similar to that obtained previously for avibactam ( $21,580 \pm 2,200$  M $^{-1}$   $s^{-1}$ ) (3, 9).

To determine the rate at which relebactam is released from KPC-2, or  $k_{off}$ , KPC-2 (1  $\mu$ M) was preincubated with relebactam (17.25  $\mu$ M) at a concentration that resulted in an initial velocity value of  $\sim 0$   $\mu$ M/s for 50  $\mu$ M nitrocefirin hydrolysis. The reaction mixture was diluted 1:10,000, and progress curves measuring the recovery of nitrocefirin hydrolysis were determined. The progress curves were fit to a single exponential equation to obtain  $k_{off}$  values (Fig. 2D). The  $k_{off}$  value of KPC-2 for relebactam was  $0.00020 \pm 0.00002$   $s^{-1}$ , which was similar to the reported  $k_{off}$  value of KPC-2 for avibactam of  $0.00014$   $s^{-1}$  (Table 3) (3).

The turnover number or partition ratio ( $k_{cat}/k_{inact}$ ), which is the amount of inhibitor required to reduce the initial velocity of a  $\beta$ -lactamase by  $\geq 90\%$ , was determined for



**FIG 1** Chemical structures of the compounds used in this study; the R1 side chains of the DBOs are highlighted in pink.

KPC-2 with relebactam. Only one molecule of relebactam was turned over by KPC-2 before the  $\beta$ -lactamase was inactivated (Table 3). Relebactam is as potent as avibactam when measuring  $k_{\text{cat}}/k_{\text{inact}}$  (9).

**Mapping the intermediates of inactivation by relebactam.** To determine if any intermediates are formed (e.g., the loss of sulfate from relebactam when it is bound to KPC-2) or if hydrolysis of relebactam occurs upon the interaction of relebactam with KPC-2, timed mass spectrometry (MS) was conducted. Five-minute and 24-h preincubations revealed that relebactam binds as the whole molecule plus  $348 \pm 5$  Da to the KPC-2 carbapenemase and is not hydrolyzed or modified (Fig. 3). The lack of modification of relebactam upon interaction with KPC-2 is different from what was observed with avibactam. Unlike with relebactam, when KPC-2 reacts with avibactam, avibactam loses its sulfate and eventually deacylates (3).

**Modeling suggests that the positioning of active-site water molecules improves the stability of the KPC-2–relebactam complex.** To understand why relebactam is not susceptible to desulfation during interactions with KPC-2 while avibactam is, relebactam was docked into the KPC-2 active site and the model was compared to the KPC-2–avibactam crystal structure (10). In addition, a molecular dynamics simulation (MDS) was conducted on both complexes for 0.2 ns.

Relebactam adopted two primary conformations when docked into the KPC-2 active site; the most energetically favorable complex was chosen for further analysis. Representations of the KPC-2–avibactam crystal structure (PDB accession number [4ZBE](#)) and the KPC-2–relebactam molecular models are presented in Fig. 4A. During the course of MDS, the positions of two site water molecules (W1 and W2) were found to be closer to the DBO sulfate in the KPC-2–avibactam crystal structure than the KPC-2–relebactam model (Fig. 4A and B). Notably, the deacylation water held via hydrogen bonding interactions to E166 and N170 remained in a similar position in the KPC-2–avibactam structure and the KPC-2–relebactam model throughout the MDS (data not shown). In the KPC-2–avibactam structure, W1 maintains a close distance ( $\approx 3$  Å) to the sulfate

**TABLE 1** Modal MICs for 101 clinical isolates of *Enterobacteriaceae* producing KPCs and 2 control strains<sup>a</sup>

Strain	Modal MIC (mg/liter)			
	CAZ	CAZ-AVI	IMI	IMI-REL
<i>Klebsiella pneumoniae</i> KPC-2	64	1	16	1
<i>E. coli</i> DH10B/pBR322-catI-bla <sub>KPC-2</sub>	64	1	8	0.5
<i>K. pneumoniae</i> VA-398	64	1	2	0.25
<i>K. pneumoniae</i> VA-400	>128	1	8	0.25
<i>K. pneumoniae</i> VA-184	>128	2	8	0.5
<i>K. pneumoniae</i> VA-237	128	0.25	8	0.125
<i>K. pneumoniae</i> VA-267	>128	1	8	0.125
<i>K. pneumoniae</i> VA-357	>128	2	16	0.25
<i>K. pneumoniae</i> VA-360	>128	4	64	0.25
<i>K. pneumoniae</i> VA-362	64	1	16	0.5
<i>K. pneumoniae</i> VA-364	64	1	32	1
<i>K. pneumoniae</i> VA-367	>128	2	2	0.25
<i>K. pneumoniae</i> VA-368	>128	1	4	0.25
<i>K. pneumoniae</i> VA-373	32	1	4	0.5
<i>K. pneumoniae</i> VA-401	>128	4	64	0.5
<i>K. pneumoniae</i> VA-376	>128	2	32	0.5
<i>K. pneumoniae</i> VA-378	>128	1	16	0.5
<i>K. pneumoniae</i> VA-380	>128	2	16	0.5
<i>K. pneumoniae</i> VA-383	>128	1	8	0.5
<i>K. pneumoniae</i> VA-384	>128	1	64	1
<i>K. pneumoniae</i> VA-387	>128	2	128	2
<i>K. pneumoniae</i> VA-402	>128	4	16	0.25
<i>K. pneumoniae</i> VA-389	>128	2	8	0.25
<i>K. pneumoniae</i> VA-390	64	1	64	0.25
<i>K. pneumoniae</i> VA-391	>128	4	8	0.125
<i>K. pneumoniae</i> VA-392	>128	1	16	0.125
<i>K. pneumoniae</i> VA-394	>128	2	8	0.25
<i>K. pneumoniae</i> VA-395	>128	2	16	0.25
<i>K. pneumoniae</i> VA-396	128	1	32	0.125
<i>K. pneumoniae</i> VA-413	64	1	64	0.25
<i>K. pneumoniae</i> VA-414	>256	4	64	0.125
<i>K. pneumoniae</i> VA-416	>256	4	64	0.5
<i>K. pneumoniae</i> VA-417	256	2	16	0.5
<i>Enterobacter cloacae</i> VA-407	>256	8	16	0.25
<i>Enterobacter aerogenes</i> VA-415	>256	2	8	0.25
<i>E. coli</i> S246579	256	1	16	0.5
<i>E. coli</i> X173170	64	1	8	0.5
<i>E. cloacae</i> M627513	256	2	4	0.5
<i>E. aerogenes</i> M2084230	128	1	8	0.5
<i>Citrobacter freundii</i> M5092134	64	1	4	0.5
<i>E. cloacae</i> M2051712	16	1	4	0.25
<i>C. freundii</i> M2295131	64	1	2	0.25
<i>Citrobacter koseri</i> M3301980	128	2	8	0.5
<i>E. aerogenes</i> M6315040	256	1	8	0.25
<i>E. coli</i> M7123031	16	0.5	4	0.25
<i>E. coli</i> M7123093	16	0.5	4	0.25
<i>E. cloacae</i> 1225904003	64	2	128	1
<i>E. cloacae</i> F17482	>256	4	128	0.5
<i>Klebsiella oxytoca</i> 1223805125	64	1	4	0.25
<i>E. cloacae</i> 1220402851	>256	4	8	0.25
<i>K. pneumoniae</i> VA 403	>512	1	32	0.25
<i>K. pneumoniae</i> VA 404	256	0.5	8	0.25
<i>K. pneumoniae</i> VA 408	256	1	8	0.125
<i>K. pneumoniae</i> VA 409	256	2	16	<0.06
<i>K. pneumoniae</i> VA 410	128	<0.06	4	0.125
<i>K. pneumoniae</i> VA 412	256	0.5	8	0.125
<i>K. pneumoniae</i> VA 361	256	0.5	8	0.25
<i>K. pneumoniae</i> VA 406	256	32	>128	2
<i>K. pneumoniae</i> VA 375	256	1	8	0.25
<i>K. pneumoniae</i> UNC 001	512	2	4	0.25
<i>K. pneumoniae</i> UNC 002	256	1	4	0.25
<i>K. pneumoniae</i> UNC 005	256	2	16	1
<i>K. pneumoniae</i> UNC 008	512	1	16	0.25

(Continued on next page)

**TABLE 1** (Continued)

Strain	Modal MIC (mg/liter)			
	CAZ	CAZ-AVI	IMI	IMI-REL
<i>K. pneumoniae</i> UNC 010	512	1	8	0.25
<i>K. pneumoniae</i> UNC 011	64	0.25	0.5	0.25
<i>K. pneumoniae</i> UNC 012	128	1	4	0.25
<i>K. pneumoniae</i> UNC 015	128	1	16	0.5
<i>K. pneumoniae</i> UNC 016	512	1	2	0.25
<i>K. pneumoniae</i> UNC 018	256	1	8	0.5
<i>K. pneumoniae</i> UNC 020	256	0.125	4	0.125
<i>K. pneumoniae</i> UNC 027	>512	1	8	0.25
<i>K. pneumoniae</i> UNC 030	256	2	4	0.5
<i>K. pneumoniae</i> UNC KPC 171	256	1	2	0.125
<i>K. pneumoniae</i> 120/1020,2	256	0.5	32	0.25
<i>K. pneumoniae</i> 140/1040,2	256	0.5	32	0.25
<i>K. pneumoniae</i> 160/1080,2	256	0.5	4	0.125
<i>K. pneumoniae</i> 300/1240,2	>512	0.25	8	0.125
<i>K. pneumoniae</i> 320/1260	32	0.5	8	0.25
<i>K. pneumoniae</i> 361/1301,2	512	1	8	0.5
<i>K. pneumoniae</i> 440/1360	256	1	16	0.125
<i>K. pneumoniae</i> 540/1460	512	0.5	4	0.25
<i>K. pneumoniae</i> 600/1500	512	2	16	0.125
<i>K. pneumoniae</i> 620/1520	256	1	8	0.125
<i>K. pneumoniae</i> 644/1566	128	1	64	0.5
<i>K. pneumoniae</i> 646/1568	256	1	8	0.25
<i>K. pneumoniae</i> 647/1569	512	2	16	2
<i>K. pneumoniae</i> 648/1570	32	0.25	0.5	0.25
<i>K. pneumoniae</i> 649/1571	32	0.5	8	0.25
<i>K. pneumoniae</i> 651/1573	512	1	8	0.25
<i>K. pneumoniae</i> 660/1586	512	<0.06	0.5	0.125
<i>K. pneumoniae</i> 665/1593	256	0.5	64	0.5
<i>K. pneumoniae</i> 666/1594	256	0.5	8	0.125
<i>K. pneumoniae</i> 670/1598	512	1	8	2
<i>K. pneumoniae</i> 671/1599	256	2	128	1
<i>K. pneumoniae</i> 672/1600	256	0.5	16	1
<i>K. pneumoniae</i> 674/1603	256	2	8	0.25
<i>K. pneumoniae</i> 676/1606	>512	1	>128	2
<i>K. pneumoniae</i> 677/1607	256	0.125	8	0.25
<i>K. pneumoniae</i> 679/1608	256	1	4	0.5
<i>K. pneumoniae</i> 681/1612	64	0.5	16	0.5
<i>K. pneumoniae</i> 682/1613	256	0.5	64	0.5
<i>K. pneumoniae</i> 686/1617	128	0.5	16	0.5
<i>K. pneumoniae</i> 691/1633	256	<0.06	2	0.125

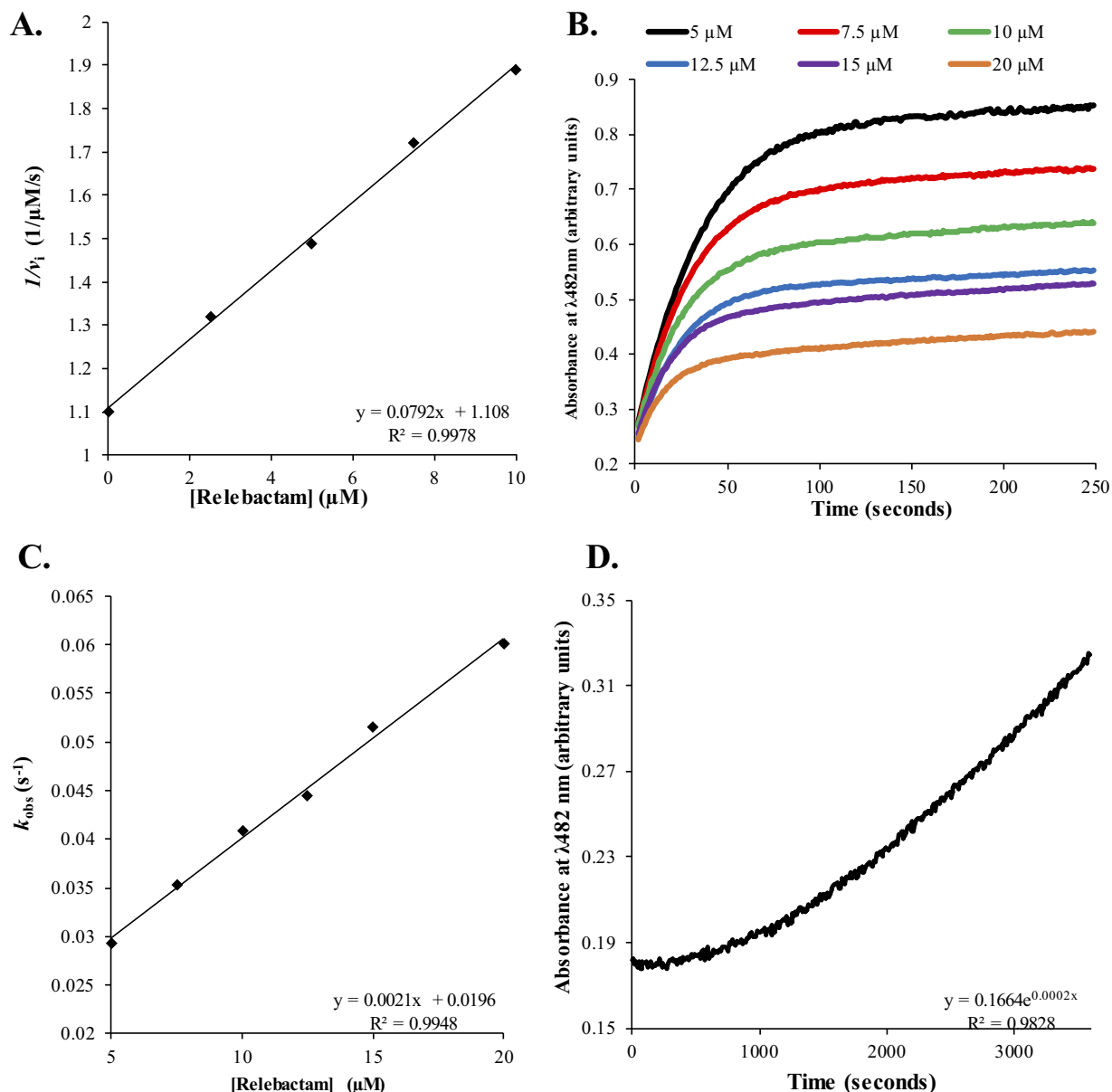
<sup>a</sup>Data are from three experiments. The control strains were *Klebsiella pneumoniae* KPC-2 and *E. coli* DH10B/pBR322-*catI-bla<sub>KPC-2</sub>*. CLSI breakpoints for ceftazidime (for both ceftazidime and ceftazidime-avibactam) and imipenem (for both imipenem and imipenem-relebactam) were used to differentiate susceptibility versus resistance. CAZ, ceftazidime; AVI, avibactam; IMI, imipenem; REL, relebactam.

group and W2 is recruited in the proximity of the N6 sulfate group as well (Fig. 4B). However, in the KPC-2–relebactam model, the water molecules move greater than 5 to 6 Å from the N6 and/or sulfate group of relebactam (Fig. 4B). Hydrolysis is involved in both the desulfation and deacylation of avibactam from KPC-2; the mechanism of desulfation remains to be elucidated.

**TABLE 2** Rates of susceptibility of the isolates tested

Antibiotic(s) <sup>a</sup>	No. of isolates with the following susceptibility/total no. of isolates tested (%):			
	Nonsusceptible	Susceptible	Intermediate	Resistant
CAZ	103/103 (100)	0/103 (0)	0/103 (0)	103/103 (100)
CAZ-AVI	0/103 (0)	103/103 (100)	0/103 (0)	0/103 (0)
IMI	94/103 (91)	9/103 (9)	17/103 (16)	77/103 (75)
IMI-REL	0/103 (0)	103/103 (100)	0/103 (0)	0/103 (0)

<sup>a</sup>CAZ, ceftazidime; AVI, avibactam; IMI, imipenem; REL, relebactam.



**FIG 2** (A) Determination of the  $K_{i, \text{app}}$  value of relebactam for KPC-2 by using increasing concentrations of relebactam and inhibiting nitrocefins hydrolysis. The inverse initial velocity ( $v_i$ ) versus the concentration of relebactam fit to a linear equation,  $K_{i, \text{app}}$  observed = y intercept/slope, is plotted.  $K_{i, \text{app}}$  observed was adjusted for the use of nitrocefins to obtain the  $K_{i, \text{app}}$  value (Table 3). (B) Progress curves showing inhibition of nitrocefins hydrolysis by KPC-2 with increasing concentrations of relebactam. The absorbance at a  $\lambda$  of 482 nm versus time is plotted. To obtain  $k_{\text{obs}}$  values, progress curves were fit to  $y = V_f \cdot x + (V_0 - V_f) \cdot [1 - \exp(-k_{\text{obs}} \cdot x)]/k_{\text{obs}} + A_0$ , where  $V_f$  is the final velocity,  $V_0$  is the initial velocity, and  $A_0$  is initial absorbance at 482 nm. (C) Determination of the  $k_2/K$  value of relebactam for KPC-2 by using increasing concentrations of relebactam and inhibiting nitrocefins hydrolysis.  $k_{\text{obs}}$  values versus the concentration of relebactam fit to a linear equation,  $k_2/K$  observed = slope, are plotted.  $k_2/K$  observed was adjusted for the use of nitrocefins to obtain the  $k_2/K$  value (Table 3). (D) Progress curves showing the recovery of nitrocefins hydrolysis by KPC-2 after inhibition by relebactam. The absorbance at a  $\lambda$  of 482 nm versus time is plotted. Progress curves were fit to a single exponential equation to obtain  $k_{\text{off}}$  values (Table 2).

Based on our observations, we hypothesize that the lack of critical water molecules near relebactam sulfate in the KPC-2–relebactam complex may play a role in relebactam's stability observed by mass spectrometry.

**Conclusions.** The favorable inhibitory kinetic profile of relebactam against the KPC-2  $\beta$ -lactamase is in agreement with the robust activity profile of relebactam against clinical isolates of KPC-producing *Enterobacteriaceae*, which is similar overall to the activity profiles of avibactam and the ceftazidime-avibactam combination. The one observable difference between relebactam and avibactam is that under our experi-

**TABLE 3** Kinetic parameters against KPC-2 using relebactam

Kinetic parameter	Value for KPC-2
$K_{i, app}$ ( $\mu\text{M}$ )	$2.3 \pm 0.3$
$k_2/K$ ( $\text{M}^{-1} \text{s}^{-1}$ )	$24,750 \pm 2,475$
$k_{off}$ ( $\text{s}^{-1}$ )	$0.00020 \pm 0.00002$
Half-life <sup>a</sup> (min)	$58 \pm 6$
$K_d^b$ (nM)	$8 \pm 1$
$k_{cat}/k_{inact}$	1

<sup>a</sup>Residence time.

<sup>b</sup> $K_d$ , dissociation constant.

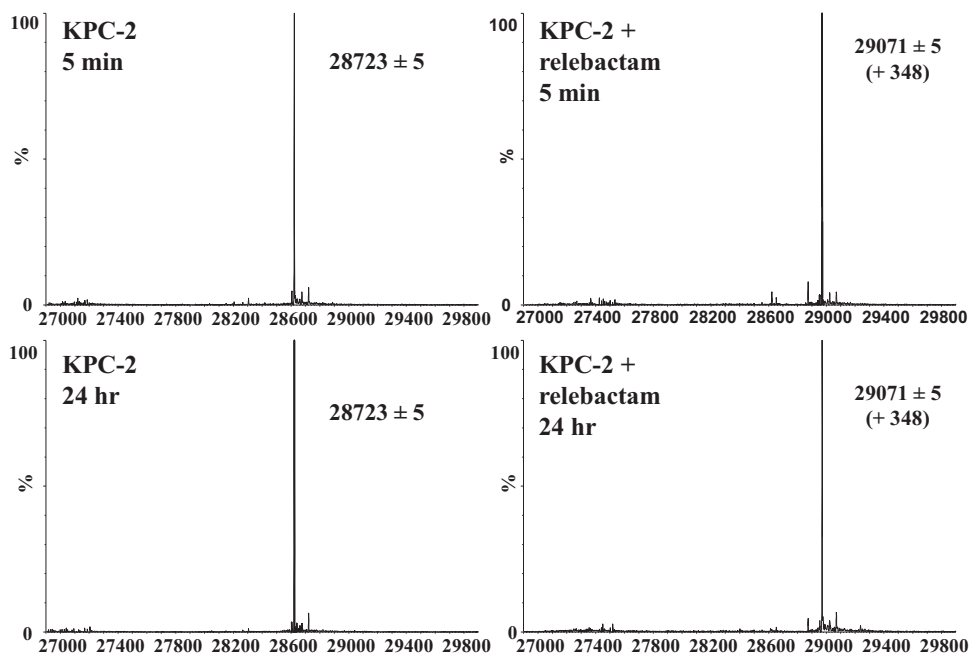
mental conditions, relebactam does not desulfate or deacylate upon reacting with KPC-2. The time scale for desulfation of avibactam by KPC-2 is within a bacterial cell replication cycle; however, deacylation of avibactam requires hours, and the complete conversion to the apo-enzyme approximates nearly 24 h. Presently, the implications of desulfation and deacylation are not clear; however, with selective pressure, one can only predict that KPC-2 may evolve to desulfate/deacylate avibactam at a higher rate. In closing, it is also possible that the differential placement of W1 and W2 within the active site may have a significant impact in the future. Once imipenem-relebactam becomes commercially available, we will develop a greater understanding of whether desulfation of DBOs by  $\beta$ -lactamases is a clinically relevant phenotype.

**MATERIALS AND METHODS**

**Isolates.** The strains used as controls for susceptibility testing, *Klebsiella pneumoniae* expressing  $bla_{KPC-2}$  and *Escherichia coli* with pBR322-*catI*- $bla_{KPC-2}$  as well as the strain used for KPC-2 protein purification, *E. coli* Origami 2 DE3 with pET24(a)+ $bla_{KPC-2}$ , were previously described (11, 12). The clinical isolates tested in this study were obtained from previous studies as well as from a collection at the University of North Carolina (13, 14).

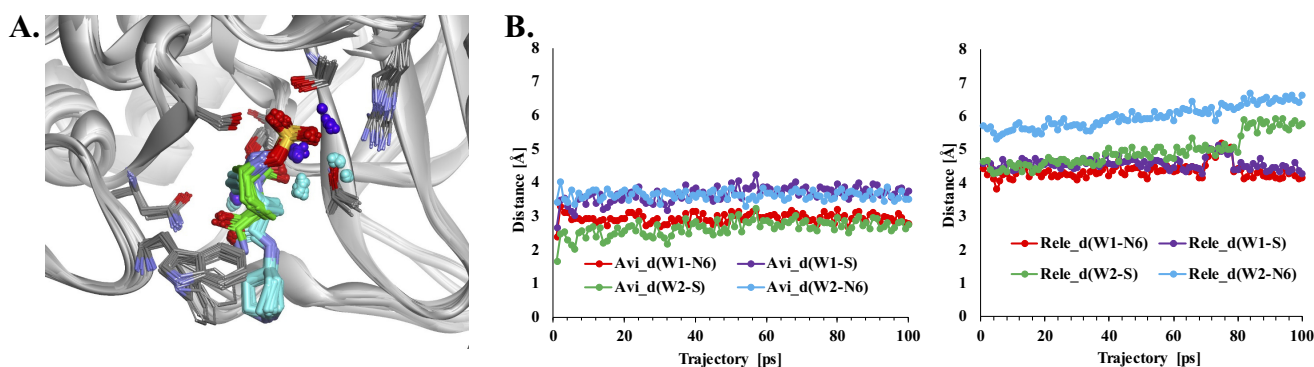
**Compounds.** Relebactam, imipenem, and avibactam were given to us as part of a Merck investigator studies program (MISP; grant number 53544); the source of avibactam was Advanced ChemBlocks. Nitrocefim was purchased from Oxoid-Remel, and ceftazidime was obtained from Sigma.

**In vitro susceptibility test methods.** The MICs for the isolates were determined by the Mueller-Hinton (MH) agar dilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines as previously described (15). Relebactam and avibactam were tested at 4 mg/liter in combination with increasing concentrations of imipenem and ceftazidime, respectively.



**FIG 3** Mass spectra of KPC-2 after 5 min and 24 h of preincubation with and without relebactam. Numbers on the x axis indicate mass in atomic mass units (amu).





**FIG 4** (A) Superimposition of the conformations collected during MDS of relebactam (cyan) and avibactam (green) in the KPC-2 active site displaying water molecules (cyan, KPC-2–relebactam model; green, KPC-2–avibactam model). (B) A graphical representation of the distance (in angstroms) of active-site water molecules (W1 and W2) from the N6 and sulfate (S) of avibactam (Avi) and relebactam (Rele) during the 100-ps MDS trajectory in the KPC-2–DBO complexes reveals that W1 and W2 are closer to the DBO sulfate in avibactam ( $\sim 2$  to  $4$  Å) than in relebactam ( $\sim 4$  to  $7$  Å). The d in \_d stands for distance.

**Purification and steady-state kinetic analysis with KPC-2.** The KPC-2  $\beta$ -lactamase was purified as previously described (1). Kinetics were carried out on an Agilent 8453 diode array spectrophotometer in 10 mM phosphate-buffered saline (PBS; pH 7.4) at room temperature. Determination of the values of the kinetic constants apparent  $K_i$  ( $K_{i,app}$ ),  $k_2/K_1$ ,  $k_{cat}/k_{inact}$ , and  $k_{off}$  was previously described (17).

**ESI MS.** To assess the nature of any intermediates of relebactam formed during the reaction with KPC-2, 10  $\mu$ M KPC-2 was incubated with 10  $\mu$ M relebactam for set times (i.e., 5 min and 24 h) at room temperature in 10 mM PBS, pH 7.4. Reactions were terminated by the addition of 0.1% formic acid and 1% acetonitrile. Electrospray ionization (ESI) mass spectrometry (MS) was performed on a Waters SynaptG2-Si quadrupole-time of flight mass spectrometer equipped with a LockSpray dual electrospray ion source and an Acquity H class ultraperformance liquid chromatograph, as previously described (18).

**Molecular modeling.** To obtain insight into the interactions between KPC-2 and relebactam, a molecular model of KPC-2 with relebactam was constructed using the crystal coordinates of KPC-2 (PDB accession number 2OV5) and Discovery Studio (D.S.; version 4.1; Biovia, Accelrys Inc., San Diego, CA) molecular modeling software as previously described (8, 19). Relebactam was constructed using the Fragment Builder tools, minimized, and automatically docked into the active site of KPC-2 using the LibDock module of D.S. software. The crystallographic waters were removed, the complex was solvated, the active-site crystallographic waters were added back, and the complex was minimized using the Conjugate gradient with the SHAKER algorithm. To equilibrate the complex, a 0.2-ns molecular dynamics simulation (MDS) was performed. On the basis of the calculated energies, the most energetically favorable conformation was chosen, and the complex with KPC-2 was created.

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