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Inhibitor Resistance in the KPC-2 β-Lactamase, a Preeminent Property of This Class A β-Lactamase▽

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As resistance determinants, KPC β-lactamases demonstrate a wide substrate spectrum that includes carbapenems, oxyimino-cephalosporins, and cephamycins. In addition, clinical strains harboring KPC-type β-lactamases are often identified as resistant to standard β-lactam–β-lactamase inhibitor combinations in susceptibility testing. The KPC-2 carbapenemase presents a significant clinical challenge, as the mechanistic bases for KPC-2-associated phenotypes remain elusive. Here, we demonstrate resistance by KPC-2 to β-lactamase inhibitors by determining that clavulanic acid, sulbactam, and tazobactam are hydrolyzed by KPC-2 with partition ratios (k_{cat}/k_{inact} ratios, where k_{inact} is the rate constant of enzyme inactivation) of 2,500, 1,000, and 500, respectively. Methylidene penems that contain an sp^2-hybridized C_3 carboxylate and a bicyclic R1 side chain (dihydropyrazolo[1,5-c][1,3]thiazole [penem 1] and dihydropyrazolo[5,1-c][1,4]thiazine [penem 2]) are potent inhibitors: K_m of penem 1, 0.06 ± 0.01 μM, and K_m of penem 2, 0.006 ± 0.001 μM. We also demonstrate that penems 1 and 2 are mechanism-based inactivators, having partition ratios (k_{cat}/k_{inact} ratios) of 250 and 50, respectively. To understand the mechanism of inhibition by these penems, we generated molecular representations of both inhibitors in the active site of KPC-2. These models (i) suggest that penem 1 and penem 2 interact differently with active site residues, with the carbonyl of penem 2 being positioned outside the oxyanion hole and in a less favorable position for hydrolysis than that of penem 1, and (ii) support the kinetic observations that penem 2 is the better inhibitor (k_{inact}/K_m = 6.5 ± 0.6 μM^{-1} s^{-1}). We conclude that KPC-2 is unique among class A β-lactamases in being able to readily hydrolyze clavulanic acid, sulbactam, and tazobactam. In contrast, penem-type β-lactamase inhibitors, by exhibiting unique active site chemistry, may serve as an important scaffold for future development and offer an attractive alternative to our current β-lactamase inhibitors.

In *Klebsiella pneumoniae*, β-lactam resistance is mediated predominantly by class A SHV, TEM, and CTX-M β-lactamases (7, 35). Single amino acid substitutions in the SHV and TEM β-lactamases can drastically alter the substrate profiles of the enzymes and confer resistance to extended-spectrum cephalosporins and β-lactamase inhibitors (5, 12, 34, 36). β-Lactamases with altered substrate profiles (i.e., extended-spectrum or inhibitor-resistant β-lactamases) have significantly challenged the clinician's approach to the treatment of serious infectious diseases (36). Thus, the search for effective mechanism-based inhibitors of novel β-lactamases merits significant effort (8, 9, 32).

First identified in *K. pneumoniae*, KPC class A β-lactamases threaten the use of all current β-lactam antibiotics (57). These β-lactamase enzymes are present in an increasing number of bacterial genera, becoming the major carbapenemase expressed by Gram-negative pathogens (e.g., *Enterobacter* spp., *Escherichia coli*, *Citrobacter freundii*, *Pseudomonas* spp., *Serratia marcescens*, *Proteus mirabilis*, and *Salmonella enterica*) in the United States (3, 10, 11, 16, 17, 25, 37, 45, 49, 53, 59). Moreover, KPC β-lactamases are becoming geographically widespread (having been detected, e.g., in the United States, China, France, Colombia, Greece, Sweden, Norway, Argentina, the United Kingdom, Israel, Brazil, Puerto Rico, Canada, Ireland, Trinidad and Tobago, Poland, Italy, and Finland) (1, 2, 15, 23, 24, 29–31, 33, 38, 39, 42, 50, 51, 53, 55, 57). Evidence suggests that many *K. pneumoniae* strains in the United States harboring KPCs are genetically related (19).

Why are KPC β-lactamases so problematic? KPC-2 has an overall structure similar to those of other class A enzymes, and interestingly, this β-lactamase has only 50% protein sequence conservation compared to CTX-M-1, 39% compared to SHV-1, and 35% compared to TEM-1. KPC-2 is more like other class A carbapenemases, having 55% identity to NmcA and Imi-1, 63% identity to Sfc-1, and 57% identity to Sme-1. The KPC-2 β-lactamase possesses a large and shallow active site, allowing it to accommodate "bulkier" β-lactams (26). As a result of these structural characteristics, KPC-2 is regarded as a versatile β-lactamase (37); it is a penicillinase, carbapenemase, and cephamycinase and an extended-spectrum β-lactamase (57, 58). Microbiologists and clinicians have observed that many bla_{KPC-2}-containing strains are resistant to β-lactam–β-lactamase inhibitor combinations (6, 19, 50, 54, 55, 59). According to Clinical and Laboratory Standards Institute (CLSI) breakpoints, bla_{KPC-2}-carrying clinical strains for which the MICs of amoxicillin-clavulanic acid are ≥32/16 mg/liter and those of piperacillin-tazobactam are ≥128/4 mg/liter are resistant (14, 58). These observations led us to examine the kinetic

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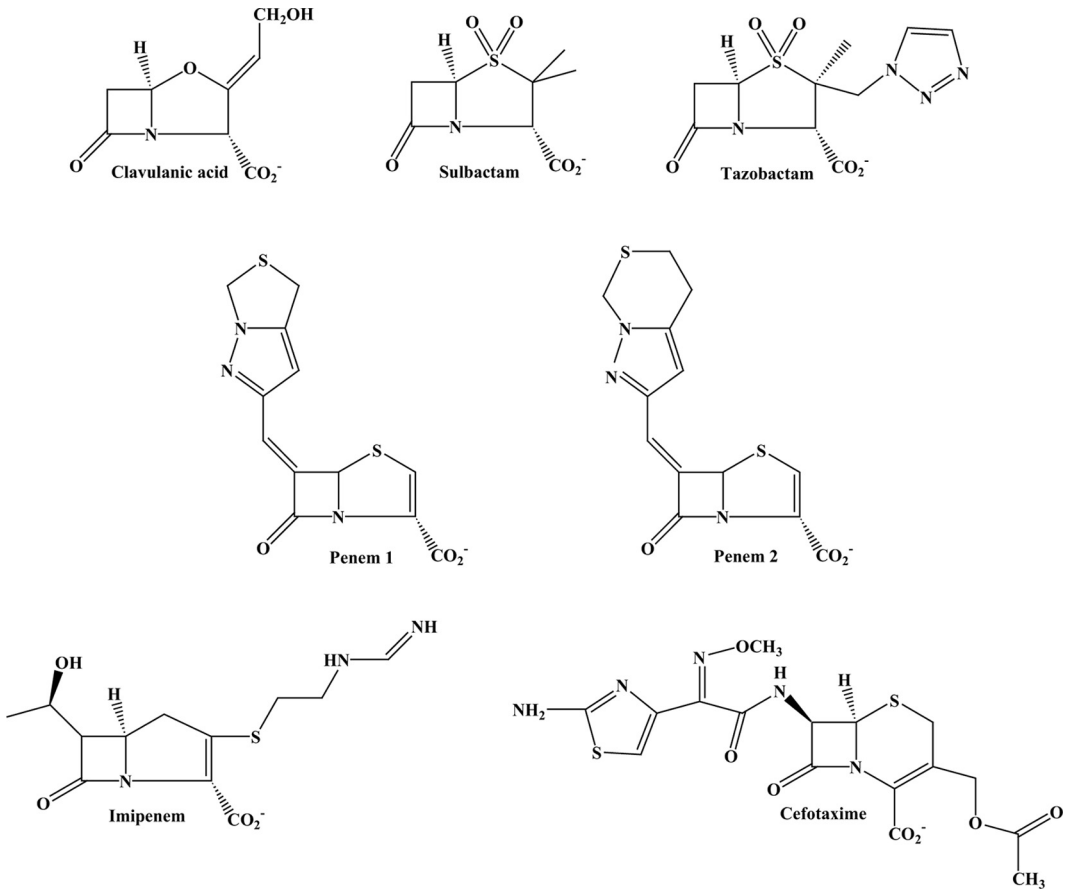

FIG. 1. Chemical structures of the classical β-lactamase inhibitors, the novel penem β-lactamase inhibitors, cefotaxime, and imipenem.

properties of the KPC-2 β-lactamase tested against commercially available and novel inhibitors.

A β-lactamase inhibitor demonstrating an affinity in the nanomolar range for KPC-2 and other class A carbapenemases would be an important addition to our therapeutic armamentarium. Thus, we wondered if penem inhibitors that possess an sp^2-hybridized C_3 carboxylate (a property resembling a characteristic of carbapenems), a complex and reactive R1 side chain, and inactivation chemistry different from that of clavulanic acid could be exploited to inhibit KPC enzymes (41). The methylidene inhibitors penem 1 and penem 2 have dihydropyrazolo[1,5-c][1,3]thiazole and dihydropyrazolo[5,1-c][1,4] thiazine moieties, respectively (see Fig. 1). These penems demonstrate similar levels of *in vivo* efficacy in mice and have been shown to be effective inhibitors of several class A, C, and D β-lactamases (4, 43, 46–48, 52).

In this paper, we show why *K. pneumoniae* containing bla_{KPC-2} and an *E. coli* laboratory strain harboring bla_{KPC-2} are not susceptible to the commercially available β-lactamase inhibitors. Our results demonstrated that clavulanic acid, sulbactam, and tazobactam are hydrolyzed by the KPC-2 β-lactamase. 6-Methylidene penems with complex fused bicyclic R1 side chains are better inhibitors because they possess greater affinity for the active site, have low K_ms, and act as mechanism-based inactivators.

MATERIALS AND METHODS

Bacterial strains and plasmids. *K. pneumoniae* possessing bla_{KPC-2} and *E. coli* containing bla_{KPC-2} in a pBR322-*catI* vector (pBR322-*catI*-bla_{KPC-2}) were kind gifts from Fred Tenover of the Centers for Disease Control and Prevention (Atlanta, GA) (58). We verified the sequence of bla_{KPC-2} in the pBR322-*catI* vector. All DNA sequencing reactions were conducted by the Genomics Core Facility at Case Western Reserve University (Cleveland, OH). *E. coli* DH10B cells (Invitrogen, Carlsbad, CA) were used as a host strain for the pBR322-*catI*-bla_{KPC-2} plasmid.

Antibiotic susceptibility. *K. pneumoniae* 1534 possessing bla_{KPC-2}, *E. coli* DH10B, and *E. coli* DH10B expressing bla_{KPC-2} were phenotypically characterized using lysogeny broth agar dilution MICs according to CLSI guidelines (13). MICs of antibiotics were determined using a Steers replicator that delivered 10-μl samples containing 10^4 CFU per spot. For determination of the β-lactamase inhibitor MICs, ampicillin was maintained at a constant concentration of 50 mg/liter and clavulanic acid and sulbactam concentrations were increased (43). In testing of piperacillin-tazobactam, the combination was used at an 8:1 ratio. Finally, penem 1 and penem 2 were maintained at 4 mg/liter while the concentrations of piperacillin, imipenem, and cefotaxime were increased.

Sulbactam was a gift from Thomas Gootz, formerly of Pfizer (Groton, CT). Clavulanic acid was obtained from GlaxoSmithKline (Brentford, United Kingdom). Tazobactam was given to us by Wyeth Pharmaceuticals (Pearl River, NY). Penem 1 and penem 2 were kindly provided by Tarek Mansour of Wyeth Pharmaceuticals (Pearl River, NY). The details of the chemical synthesis of penems 1 and 2 are summarized by Venkatesan et al. (47). Imipenem was purchased from U.S. Pharmacopeia (Rockville, MD). Piperacillin and cefotaxime were purchased from Sigma (St. Louis, MO). The chemical structures of compounds used in this study are shown in Fig. 1.

β-Lactamase purification. The KPC-2 β-lactamase was purified from *E. coli* DH10B cells carrying the pBR322-*catI*-bla_{KPC-2} plasmid. The cells were grown for 18 h in 500 ml of superoptimal broth (SOB) containing 20 mg/liter chloram-

phenicol (Sigma) at 37°C with shaking. Cells were pelleted by centrifugation at 5000 × *g* for 15 min. Cell pellets were frozen for 18 h at −20°C and resuspended in 50 mM Tris-Cl, pH 7.4, and periplasmic proteins were released using stringent periplasmic fractionation with 40 mg/liter lysozyme followed by the addition of 2.0 mM EDTA. The supernatant was further enriched for β-lactamase by using preparative isoelectric focusing as described previously (27, 43). A second purification step was performed using fast protein liquid chromatography (FPLC) with a size exclusion HiLoad 16/60 Superdex-75 column (GE Healthcare Life Sciences, Uppsala, Sweden) on the ÄKTA P-900 system (GE Healthcare Life Sciences). Proteins were eluted with 10 mM phosphate-buffered saline (PBS) at pH 7.4, and fractions were analyzed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were resolved on an SDS-PAGE gel with 5% acrylamide for stacking and 12% acrylamide for separation and stained with Coomassie brilliant blue R250. A final purification step using anion-exchange chromatography with a HiTrap Q HP column (GE Healthcare Life Sciences) was needed. Proteins were eluted with a salt gradient using a mixture of 50 mM Tris-Cl at pH 8.8 and 1.0 M sodium chloride at pH 8.8. The purity of each preparation was again assessed by SDS-PAGE. Protein concentrations were determined with a protein assay using bovine serum albumin as a protein standard according to the protocol of the assay system manufacturer (Bio-Rad, Hercules, CA).

ESI-MS. Electrospray ionization (ESI)-mass spectrometry (MS) analysis of the intact KPC-2 β-lactamase, purified from *E. coli* DH10B cells carrying the pBR322-*catI*-bla_{KPC-2} plasmid, was performed with a Q-STAR XL quadrupole time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a nanospray source as described previously (44). β-Lactamase enzymes (*E*) were incubated with an inhibitor (*I*) for 15 min at room temperature in 10 mM PBS, pH 7.4. The *I*:*E* ratios were greater than the turnover number at 15 min (see below). Reactions were terminated by equilibration with 0.1% trifluoroacetic acid (TFA). All samples were desalted and concentrated using a C_{18} ZipTip according to the protocol of the manufacturer (Millipore, Billerica, MA). Eluted protein samples were diluted with 50% acetonitrile and 0.1% TFA to a concentration of 10 μM and infused at a rate of 0.5 μl per min, and data were collected for 2 min. Spectra were deconvoluted using the Applied Biosystems Analyst program.

Kinetics. Steady-state kinetic parameters were determined using an 8453 diode array spectrophotometer (Agilent, Santa Clara, CA). Each assay was performed with 10 mM PBS, pH 7.4, at room temperature. In all assays, the enzyme was maintained at 10 nM while substrate concentrations were varied from 5 to 200 μM. To measure hydrolysis rates, we used the following extinction coefficients (Δε): nitrocefin (NCF) Δε, 17,400 M^{-1} cm^{-1} at 482 nm; piperacillin Δε, −820 M^{-1} cm^{-1} at 235 nm; cefotaxime Δε, −7,250 M^{-1} cm^{-1} at 262 nm; imipenem Δε, −9,000 M^{-1} cm^{-1} at 299 nm; and penem 1 Δε, −32,400 M^{-1} cm^{-1} at 290 nm. We also observed that in 10 mM PBS at room temperature, imipenem undergoes spontaneous hydrolysis. We subtracted the rate of this spontaneous hydrolysis from our measured velocity to determine the "true velocity" (*v*).

The kinetic parameters V_{max} and K_m were obtained by nonlinear least-squares fitting of the data (using a Michaelis-Menten equation) with Enzfitter (Biosoft Corporation, Ferguson, MO):

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

where [*S*] is the concentration of substrate.

Kinetic assays were performed under steady-state conditions to determine the K_ms of inhibitors for the enzyme according to a previously established model represented in equation 2 (21, 28). In every case, the enzyme concentration was maintained at 10 nM while inhibitor concentrations varied from 25 to 200 μM for clavulanic acid, 125 μM to 1.0 mM for sulbactam, 250 μM to 2.5 mM for tazobactam, 100 nM to 5 μM for penem 1, and 10 to 250 nM for penem 2. A final concentration of 100 μM NCF was used as the reporter substrate. The branched-pathway model followed by penems 1 and 2 can be represented by the following rate equation:

$$\begin{array}{ccccc} E + I & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & E{:}I \xrightarrow{k_2} E\text{-}I & \xrightarrow{k_3} & E + P' \\ & & \downarrow{\scriptstyle k_4} & & \\ & & E\text{-}I^* & \xrightarrow{k_5} & E + P'' \\ & & \downarrow{\scriptstyle k_6} & & \\ & & E\text{-}I^{**} & & \end{array} \quad (2)$$

In this model, formation of the noncovalent enzyme-inhibitor complex *E*:*I* is represented by the dissociation constant, *K*, which is equivalent to k_{-1}/k_1. k_2 is the first-order rate constant for the acylation step, or the formation of acyl enzyme *E*-*I*. k_3 is the rate constant for the hydrolysis of the *E*-*I* acyl enzyme. The rearrangement of *E*-*I* to yield *E*-*I** is represented by the rate constant k_4. The rate constant for the hydrolysis of the *E*-*I** acyl enzyme corresponds to k_5. Finally, the formation of a terminally inactivated acyl enzyme species, *E*-*I***, is represented by rate constant k_6. *P*′ and *P*″ are reaction products.

Inverse initial steady-state velocities ($1/v_0$) were plotted against the inhibitor concentration ([*I*]) to obtain a straight line. As previously established, for brief periods of observation, the k_5 step of equation 2 can be neglected (21, 28). Under these conditions, measuring the initial steady-state velocity immediately after mixing yielded a K_m for the hydrolysis reaction, or the Michaelis constant for the inhibitors. The initial velocity (v_0) measured after mixing is represented by equation 3.

$$v_0 = (V_{max}{}^*[S])/\{K_{m(NCF)}{}^*(1 + I/K_m) + [S]\} \quad (3)$$

where $K_{m(NCF)}$ is the K_m of NCF.

K_m(observed) was determined by dividing the value for the *y* intercept by the slope of the line. The data were corrected to account for the affinity of NCF for the β-lactamase:

$$K_m(\text{corrected}) = K_m(\text{observed})/\{1 + [S]/K_{m(NCF)}\} \quad (4)$$

The rate constant of enzyme inactivation, k_{inact}, was measured directly by time-dependent inactivation of the enzyme in the presence of an inhibitor. To perform this determination, a fixed concentration of enzyme (10 nM), 100 μM NCF, and increasing concentrations of an inactivator (50 to 1,250 μM for clavulanic acid, 500 μM to 5.0 mM for sulbactam, 250 μM to 2.5 mM for tazobactam, 400 nM to 20 μM for penem 1, and 100 nM to 1.5 μM for penem 2) were used in each assay. The observed rate constant for inactivation (k_{obs}) was determined by nonlinear least-squares fitting of the data using Origin 8.0 (Northampton, MA). Here, A_0 is initial absorbance, v_f is final velocity, and *t* is time:

$$A = A_0 + v_f \times t + (v_0 - v_f) \times [1 - \exp(-k_{obs} \times t)]/k_{obs} \quad (5)$$

Each k_{obs} value was plotted versus the inhibitor concentration and fit to determine k_{inact}. The kinetic parameter k_{inact} was obtained with nonlinear least-squares fitting of the data using Enzfitter (Biosoft Corporation) and the hyperbolic equation below:

$$k_{obs} = (k_{inact} \times [I])/(K_m + [I]) \quad (6)$$

k_{inact} as presented in equation 2 corresponds to $(k_2 \times k_4)/(k_2 + k_3 + k_4)$. The inhibitor efficiency expressed by the k_{inact}/K_m ratio is equivalent to $(k_2 \times k_4)/(K \times k_3)$.

Partition ratios, or turnover numbers ($t_n = k_{cat}/k_{inact}$ or k_3/k_4), for KPC-2 were obtained by incubating 1.0 μM KPC-2 with increasing concentrations of an inhibitor (1.0 to 10.0 mM for clavulanic acid, 500 μM to 5.0 mM for sulbactam, 50 μM to 1.0 mM for tazobactam, 100 μM to 1.0 mM for penem 1, and 5 to 100 μM for penem 2) at room temperature for 15 min in 10 mM PBS, pH 7.4. The ratio of inhibitor to enzyme (*I*:*E* ratio) necessary to inhibit the hydrolysis of NCF by greater than 90% was determined.

Recovery from inhibition by penem 1 was measured by incubating 1.0 μM KPC-2 with 1.0 mM penem 1 for 18 h at room temperature. A spin concentrator with a 10,000-molecular-weight cutoff was used to remove the unbound inhibitor/product from the *EI* mixture. NCF (100 μM) hydrolysis by KPC-2 was monitored for 40 min. Hydrolysis of 25 μM penem 1 by 200, 50, and 25 nM KPC-2 at room temperature was measured at A_{290} over 30 min (λ_{max} for penem 1, 290 nm).

UVD spectra. UV difference (UVD) spectra were obtained for 25 μM penem 1, 25 μM penem 1 reacted with 0.25 μM KPC-2, and 25 μM penem 1 reacted with 50 mM sodium hydroxide (NaOH) in 10 mM PBS, pH 7.4. UVD spectra were measured from wavelengths of 200 to 400 nm on an Agilent 8453 diode array spectrophotometer for 1 min at room temperature.

Molecular modeling. The crystal structure of KPC-2 (Protein Data Bank accession no. 2OV5) was used to generate molecular representations of the β-lactamase interactions with the two penem inhibitors. The applications Protein Reports and Utility Tools in Discovery Studio 2.1 molecular modeling software (Accelrys, San Diego, CA) were used to correct crystallographic disorder and prepare the protein for molecular modeling. Hydrogen atoms were added, the pH was set at 7.4, and the crystallographic waters were removed. The KPC-2 β-lactamase was immersed in a "water box" and was centered 7.0 Å away from any face of the box. Then the molecule was subjected to energy minimization in several steps using steepest-descent and conjugate gradient algorithms to reach the minimum convergence (0.02 after 10,000 iterations and a final potential

TABLE 1. MICs of β-lactams and combinations of β-lactams with commercially available β-lactamase inhibitors and penems 1 and 2

Drug or combination[a]	MIC (mg/liter) for:		
	K. pneumoniae 1534 bla_{KPC-2}	*E. coli* DH10B (pBR322) bla_{KPC-2}	*E. coli* DH10B
Ampicillin	8,192	4,096	1
Ampicillin-clavulanic acid	50/32	50/32	50/1
Ampicillin-sulbactam	50/512	50/512	50/1
Piperacillin	1,024	1,024	2
Piperacillin-tazobactam	512/64	512/64	4/0.5
Piperacillin-penem 1	16/4	32/4	1/4
Piperacillin-penem 2	32/4	64/4	4/4
Cefotaxime	16	8	0.06
Cefotaxime-penem 1	0.25/4	0.25/4	0.06/4
Cefotaxime-penem 2	0.25/4	0.5/4	0.06/4
Imipenem	8	8	0.125
Imipenem-penem 1	2/4	2/4	0.125/4
Imipenem-penem 2	2/4	2/4	0.25/4

[a] In drug combinations, ampicillin was maintained at a constant concentration of 50 mg/liter and clavulanic acid and sulbactam concentrations were increased. For piperacillin-tazobactam, the two components were increased but maintained at a ratio of 8:1. Penem 1 and penem 2 were held at a constant concentration of 4 mg/liter while the concentrations of piperacillin, imipenem, and cefotaxime were increased.

energy of −370,864 kJ/mol). Energy minimizations and molecular dynamics simulations for the enzyme and complexes were performed using force-field parameters of CHARMm and a dielectric constant of 1.0. The particle mesh Ewald (PME) method was used to treat long-range electrostatics. Bonds that involved hydrogen atoms were constrained with the SHAKE algorithm.

The penem structures were constructed using Fragment Builder tools. The CHARMm force field was applied. The protein was solvated and minimized using a Standard Dynamics Cascade protocol of Discovery Studio 2.1. The hydrolyzed penems were automatically docked into the active site of the β-lactamase using the Flexible Docking module of Discovery Studio 2.1. The protocol allowed docking of penems into the flexible active site of KPC-2. Flexible docking using the ChiFlex algorithm (40) created side chains and penem conformations, while the LibDock algorithm (18) docked the low-energy penem conformations into the active site of KPC-2. In the presence of penems, the side chains of selected active site residues were refined using the ChiRotor algorithm (40), after which a final simulated annealing and energy minimization step for each ligand conformation was carried out using CDOCKER (56).

RESULTS AND DISCUSSION

Susceptibility testing. In Table 1, we summarize the results of our susceptibility testing with β-lactams of the *K. pneumoniae* clinical isolate containing KPC-2, an *E. coli* transformant with bla_{KPC-2}, and an *E. coli* DH10B control. As expected, for the *K. pneumoniae* clinical strain and the *E. coli* strain containing bla_{KPC-2}, the MICs of all β-lactams tested were elevated (8 mg/liter for imipenem and 8,192 mg/liter for ampicillin). MICs of the β-lactam–β-lactamase inhibitor combinations for bla_{KPC-2}-expressing strains were also high (ampicillin-clavulanic acid MIC, 50/32 mg/liter; ampicillin-sulbactam MIC, 50/512 mg/liter; and piperacillin-tazobactam MIC, 512/64 mg/liter). These observations are consistent with current reports of MICs for clinical strains (6, 19, 50, 54, 55, 59). We next assayed the activities of piperacillin, cefotaxime, and imipenem in combination with penem 1 or penem 2. The lowest MICs were obtained when either penem 1 or 2 was combined with piperacillin, cefotaxime, or imipenem. Notably, penem 1 and penem 2 lower the MICs of imipenem from 8 to 2 mg/liter for bla_{KPC-2}-bearing strains. Interestingly, the most significant reduction for bla_{KPC-2}-carrying strains was seen when cefotaxime was combined with penem 1 and penem 2. MICs were lowered from 8 and 16 to 0.25 and 0.5 mg/liter, a six-doubling-dilution difference, when combined with 4 mg/liter of penems 1 and 2.

TABLE 2. KPC-2 kinetic parameters[a]

Drug	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ratio ($\mu M^{-1}\ s^{-1}$)
Piperacillin	16 ± 2	17 ± 6	1.1 ± 0.2
NCF	5 ± 1	30 ± 1	6.4 ± 0.2
Cefotaxime	120 ± 14	78 ± 5	0.6 ± 0.1
Imipenem	19 ± 1	19 ± 1	1.0 ± 0.1

[a] Values are means ± standard deviations.

Kinetics of KPC-2 with substrates and inhibitors. KPC-2 was purified, and steady-state kinetic parameters were measured (Table 2). As demonstrated previously, KPC-2 hydrolyzes piperacillin, NCF, cefotaxime, and imipenem. Consistent with the MIC data, the catalytic efficiency (k_{cat}/K_m ratio) is lowest for cefotaxime, at 0.61 $\mu M^{-1}\ s^{-1}$, and highest for NCF, at 6.4 $\mu M^{-1}\ s^{-1}$, among the substrates tested (58).

To measure the kinetic parameters of inactivation, we used NCF as the reporter substrate (Table 3). In our assays, clavulanic acid, tazobactam, and sulbactam were unable to effectively inhibit NCF hydrolysis by KPC-2 (k_{inact}/K_m ratio, <0.003 $\mu M^{-1}\ s^{-1}$ for all three inhibitors). More importantly, we also showed that KPC-2 hydrolyzes over 2,500 molecules of clavulanic acid, 1,000 molecules of sulbactam, and 500 molecules of tazobactam in 15 min. After 24 h, there is at least 50% further recovery of KPC-2 activity from inhibition by sulbactam and tazobactam at drug concentrations equal to the t_n measured at 15 min. By 72 h after the addition of the drug, KPC-2 partially recovers (~5%) from inhibition by clavulanic acid.

In contrast, we observed that the k_{inact}/K_m ratios for penems 1 and 2 with the KPC-2 β-lactamase were 0.18 and 6.5 $\mu M^{-1}\ s^{-1}$, respectively; these values were in accordance with the lower MIC determinations for penems 1 and 2. Despite significantly lower MICs of combinations with these penems than of combinations with other β-lactamase inhibitors, KPC-2 also hydrolyzed 250 molecules of penem 1 and 25 molecules of penem 2 in 15 min. In the case of penem 1, we were able to determine a k_{cat} of 2.5 s^{-1}. After 24 h, KPC-2 can recover from inhibition by penem 1 (~20%) or penem 2 (~30%) at inhibitor concentrations equal to the t_n measured at 15 min. Recovery after 24 h at a 1,000:1 penem 1-to-KPC-2 ratio is represented in Fig. 2D.

KPC-2, inhibitor inactivation products, and mechanism of inhibition. Timed MS was used to assess the formation of

TABLE 3. KPC-2 inhibitor kinetics with the β-lactamase inhibitors[a]

Drug	K_m (μM)	k_{inact} (s^{-1})	k_{inact}/K_m ratio ($\mu M^{-1}\ s^{-1}$)	t_n (k_{cat}/k_{inact})
Clavulanate	8.4 ± 0.8	0.027 ± 0.001	0.0036 ± 0.0004	~2,500
Sulbactam	135 ± 1	0.16 ± 0.01	0.0012 ± 0.0001	~1,000
Tazobactam	78.5 ± 7.9	0.20 ± 0.01	0.0025 ± 0.0002	~500
Penem 1	0.06 ± 0.01	0.011 ± 0.001	0.18 ± 0.02	~250
Penem 2	0.006 ± 0.001	0.039 ± 0.002	6.5 ± 0.6	~25

[a] Values are means ± standard deviations.

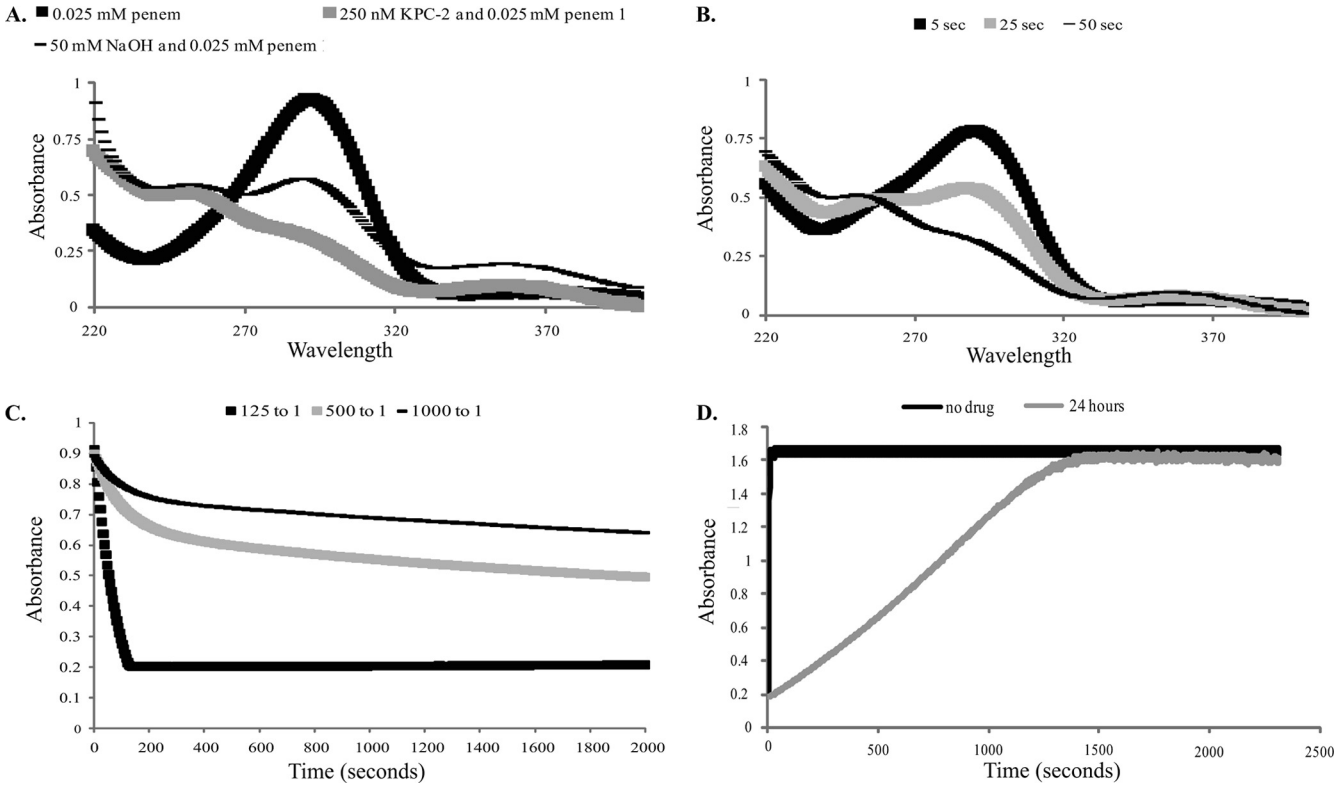

FIG. 2. (A) UVD spectra for 25 μM penem 1, a mixture of 25 μM penem 1 and 0.25 μM KPC-2, and a mixture of 25 μM penem 1 and 50 mM NaOH after 50 s of incubation. (B) UVD spectra for a mixture of 25 μM penem 1 and 0.25 μM KPC-2 monitored over periods of 5, 25, and 50 s. (C) Penem 1 hydrolysis by KPC-2 monitored at A_{290} using ratios of 125:1 (25 μM penem 1 and 200 nM KPC-2), 500:1 (25 μM penem 1 and 50 nM KPC-2), and 1,000:1 (25 μM penem 1 and 25 nM KPC-2). (D) Hydrolysis of NCF (100 μM) by 1.0 μM KPC-2 after inhibition by 1.0 mM penem 1 at a 1,000:1 inhibitor-to-enzyme ratio during 24 h of incubation at room temperature compared to hydrolysis by KPC-2 alone.

KPC-2–β-lactamase inhibitor complexes. A molecular mass of 28,477 ± 3 Da (amu) was obtained for KPC-2 alone. In preparation for MS, KPC-2 was incubated for 15 min with clavulanic acid, sulbactam, tazobactam, penem 1, or penem 2 at a concentration greater than the t_n. Notwithstanding the lower partition ratios (t_n), we were not able to trap penem 1 or penem 2 inactivating KPC-2.

We next employed timed UVD spectra to gain further insight into the process of KPC-2 inhibition by penem 1 and penem 2. The UVD spectra at 50 s for penem 1 alone, penem 1 reacted with KPC-2, and penem 1 reacted with NaOH are represented in Fig. 2A. The significant change in A_{290} when KPC-2 was incubated with penem 1 suggests that penem 1 is hydrolyzed by KPC-2 (Fig. 2B). This interpretation is supported by the UVD changes that are observed after the base hydrolysis of penem 1 (A_{290} also decreases with time).

We next studied the hydrolysis of penem 1 by KPC-2 at A_{290} (Fig. 2C). Our results show that when the I:E ratio is $>t_n$ (i.e., >250:1), a new steady state is reached. We observed that the hydrolysis of penem 1 is biphasic, with rapid initial hydrolysis ($E\text{-}I \rightarrow E + P'$; rate constant, k_3) followed by a lower steady-state rate ($E\text{-}I^* \rightarrow E + P''$; rate constant, k_5) (equation 2) after about 800 s. After 24 h at a high inhibitor-to-enzyme ratio (1,000:1), not all of penem 1 was hydrolyzed (data not shown). Remarkably, if excess penem 1 is removed, most of KPC-2's activity rapidly recovers from inhibition at a 1,000:1 ratio, with a slight lag (Fig. 2D). We also observed that there is an initial rate of hydrolysis which may be due to free enzyme (either enzyme that has not acylated or enzyme that has acylated and deacylated) (Fig. 2D). In addition, the slope of the line after the lag is lower than that for the control without penem 1, which is indicative of a terminally inactivated enzyme-inhibitor complex ($E\text{-}I^{**}$; equation 2).

To begin to understand how penem 1 and penem 2 interact with KPC-2, we modeled the penems in the active site of KPC-2. We focused upon the penems because they were the best inhibitors among those tested, including clavulanate, sulbactam, and tazobactam. Based upon our work with SHV-1 and OXA-1, we conceptualized a mechanism in which the acyl enzyme proceeds to the linear imine that ultimately undergoes 7-endo-trig cyclization to yield a cyclic enamine, the 1,4-thiazepine derivative (2, 37). Here, we focus on the deacylated forms of penems 1 and 2 before formation of the postulated seven-membered 1,4-thiazepine ring ($E + P'$).

In Fig. 3, the molecular representation of penem 1 (orange) within the active site of KPC-2 is superimposed with the representation of penem 2 (purple) in the active site. When comparing the models of the major active site interactions with penem 1 and penem 2, we note several major differences. To begin with, the carbonyl oxygen atom of penem 1 is pointing toward the oxyanion hole, whereas the carbonyl oxygen atom of penem 2 is flipped and pointing away from the oxyanion

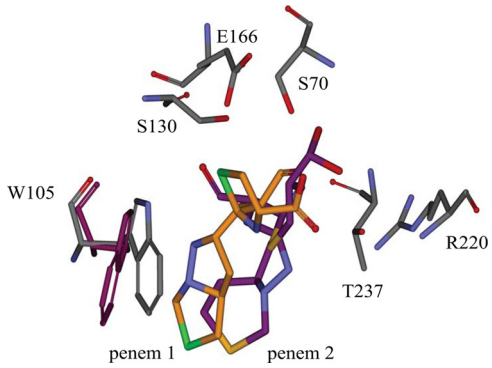

FIG. 3. Superimposition of molecular representations of penem 1 (gray-orange) and penem 2 (gray-purple) within the KPC-2 active site. Atom colors: N (blue), O (red), S (green in penem 1, yellow in penem 2).

hole. Next, we note that residues T237 and R220 have hydrogen bonding interactions with the C_3 carboxylate of penem 1, whereas neither is close enough to the C_3 carboxylate of penem 2 for hydrogen bonding interactions. Instead, the C_3 carboxylate of penem 2 is close enough for hydrogen bonding with either K234 or T235. Lastly, we observe hydrophobic interactions with a potential for π-π stacking between the W105 ring and the bicyclic ring of penem 1. However, in the penem 2 model, W105 shifts away about 50° or 2.5 Å from the penem 2 molecule. Overall, our model indicates why the penems participate in interactions leading to lower K_ms and higher k_{inact}/K_m ratios than those for the other inhibitors tested.

Conclusions. Herein, we summarize the kinetic and biochemical correlates of resistance to inhibition of KPC-2 by clavulanic acid, sulbactam, and tazobactam and we explore the turnover of two novel penems. Three important conclusions arise from the findings of our study. First, we show why the commercially available β-lactamase inhibitors are ineffective against KPC-2. To our knowledge, this ability to readily hydrolyze clavulanic acid, sulbactam, and tazobactam is very uncommon in class A enzymes (22). This unprecedented observation partly explains why MICs of β-lactam–β-lactamase inhibitor combinations are so high. For clinical isolates, this situation is compounded by the presence of multiple β-lactamases (e.g., TEM and SHV, etc). Although penem 1 and penem 2 are hydrolyzed by KPC-2 while acting as mechanism-based inactivators, they potentially offer a better alternative than the commercial inhibitors for inhibition of KPC-producing strains. We suspect that unraveling the chemistry that drives the hydrolysis of the commercially available inhibitors and penems 1 and 2 through a branched kinetic mechanism (20, 21, 28) may serve to offer new approaches to inhibiting carbapenemases.

Second, we were intrigued by the synergy between cefotaxime and penem 1 or 2. We predict that this synergy is due to the lower catalytic efficiency of the KPC-2 β-lactamase for cefotaxime (k_{cat}/K_m ratio, 0.6 ± 0.1 μM^{-1} s^{-1}) than for the other substrates tested. One wonders if combinations like this may potentially be exploited to inhibit KPC-producing bacteria in the future.

Third, penem 1 and penem 2 differ by only one carbon atom; thus, it was surprising that their turnover patterns were different. Using our model, we illustrate why these inhibitors are effective (i.e., position in the oxyanion hole) and why they may behave so differently.

We close with the thought that the future challenge in medicinal chemistry will be to anticipate the different catalytic pathways these versatile enzymes (i.e., β-lactamases) will follow as we continuously stress them with each new generation of β-lactams. We recall that both imipenem (thienamycin) and clavulanic acid were discovered in the late 1970s. It is sobering to contemplate that it has taken only 30 years to evolve a versatile β-lactamase whose carbapenem-resistant phenotype is matched by a very robust inhibitor-resistant profile. In fact, the latter may be the preeminent property of the KPC β-lactamase.

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