Potency of Vaborbactam is Less Affected than Avibactam in Strains Producing KPC-2 Mutations that Confer Resistance to Ceftazidime-Avibactam

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Key words: Vaborbactam, beta-lactamase inhibitors, ceftazidime-avibactam, KPC-2
Abstract

Resistance to ceftazidime-avibactam due to mutations in KPC genes has been reported both in vitro and in clinical settings. The most frequently reported mutation leads to the amino acid substitution D179Y in the Ω loop of the enzyme. Bacterial cells that carry mutant KPC acquire a higher level of ceftazidime resistance, become more sensitive to other cephalosporins and almost completely lose resistance to carbapenems. In this study we demonstrate that two substitutions in KPC-2, D179Y and L169P, reduce the ability of avibactam to enhance the activity of ceftazidime, cefepime or piperacillin against isogenic efflux deficient strains of *P. aeruginosa*, 8 to 32-fold and 4 to 16-fold for the D179Y and L169P variants, respectively, depending on antibiotic. In contrast, the potency of vaborbactam, the structurally unrelated beta-lactamase inhibitor that was recently approved by the FDA in combination with meropenem, is reduced no more than two-fold. Experiments with purified enzymes demonstrate that the D179Y substitution causes ~20-fold increase in the IC$_{50}$ of inhibition of ceftazidime hydrolysis by avibactam vs 2-fold for vaborbactam and that the L169P substitution has ~4.5-fold stronger effect on the affinity for avibactam compared to that for vaborbactam. In addition, D179Y and L169P variants hydrolyze ceftazidime with 10-fold and 4-fold higher efficiency, respectively, compared to wild-type KPC-2. Thus, microbiological and biochemical experiments implicate both decreased ability of avibactam to interact with KPC-2 variants and an increase in the efficiency of ceftazidime hydrolysis in resistance to ceftazidime-avibactam. These substitutions have a considerably lesser effect on interactions with vaborbactam making the meropenem-vaborbactam combination a valuable agent in managing infections due to KPC-producing carbapenem resistant Enterobacteriaceae.
Introduction

The most common mechanism of resistance to β-lactam antibiotics in Gram-negative bacteria is production of β-lactamase enzymes capable of cleaving the β-lactam ring resulting in a complete loss of activity. Inhibition of β-lactamase activity with small molecule inhibitors (BLIs) has been a broadly recognized strategy to prevent β-lactam cleavage and restore their potency (1, 2). A notable BLI is avibactam which is a potent inhibitor of numerous serine (Class A, class C and some class D) enzymes including KPC carbapenemases (3). It was approved by the FDA in 2015 in combination with ceftazidime (4). An increasing number of reports describe the successful use of ceftazidime-avibactam to treat infections caused by KPC-producing CRE (carbapenem resistant Enterobacteriaceae) (5-9).

Our own efforts led to the discovery of a structurally and mechanistically different BLI, vaborbactam, a cyclic boronate with activity against class A and class C beta-lactamases (10). Similar to avibactam, vaborbactam is a potent inhibitor of KPC enzymes (11, 12) and is capable of enhancing the activity of meropenem in vitro and in mouse infection models against KPC-producing Enterobacteriales (13, 14). In 2017 vaborbactam was approved by the FDA in combination with meropenem (15). Its utility to treat infections due to KPC-producing CRE has been demonstrated in a multinational, open-label, randomized clinical trial (16) and in a recently conducted prospective, observational study of patients with CRE infections (17).

An apparent difference between ceftazidime-avibactam and meropenem-vaborbactam is their relative ability to select for mutations in a target KPC gene. In vitro multi-step resistance development studies with the meropenem-vaborbactam combination failed to generate any target
mutations in KPC genes harbored by various clinical strains (18). No mutations in KPC genes have been reported to date in patients treated with meropenem-vaborbactam. Decreased susceptibility to meropenem-vaborbactam appears to be due to a combination of various mechanisms affecting intracellular accumulation of either meropenem or vaborbactam (porin mutations, increased efflux) (18, 19).

In contrast, in vitro single step resistance development studies using ceftazidime-avibactam as a selective agent have identified several mutations in the blaKPC-3 gene that conferred resistance to this combination (20). One of these mutations, D179Y, has been also detected worldwide in KPC-2 and KPC-3 producing clinical isolates of Enterobacteriaceae recovered from patients after treatment with the ceftazidime-avibactam combination (21-25). Importantly, this mutation concurrently resulted in restoration of susceptibility to carbapenems (24, 26, 27). Not surprisingly, strains containing KPC with the D179Y mutation are also susceptible to meropenem-vaborbactam (19). A recent report documented that treatment with meropenem-vaborbactam resulted in resolution of an infection due to KPC-producing K. pneumoniae with treatment-emergent ceftazidime-avibactam resistance (28).

It was proposed that ceftazidime-avibactam resistance conferred by the D179 substitutions can be due to stabilizing interactions (e.g. hydrogen bonds) of ceftazidime within the active site of variant beta-lactamases that prevent avibactam from binding to and inhibiting the enzyme(29, 30). However, another recent publication demonstrated a significant effect of the D179Y substitution in KPC-2 on the efficiency of avibactam acylation of the enzyme (70,000 fold decrease in $k_2/K$ value) (31).

L169P is another mutation, which is located close to D179Y in the Ω-loop region of KPC-2, that is associated with ceftazidime-avibactam resistance; it has also been recovered from a
patient treated with ceftazidime-avibactam (deposited in Genbank as KPC-35) (32, 33). Similar to the D179Y mutation, it converts clinical isolates to a carbapenem susceptible phenotype. Currently, no biochemical studies have been published on the mechanism of resistance caused by this mutation.

We initiated a series of studies focusing on the role of partner antibiotic and BLI in selecting for target-based resistance to the combination agent. In this study, we evaluated the effect of D179Y and L169P mutations on the potency of vaborbactam and avibactam to enhance the activity of various antibiotics in isogenic strains expressing KPC enzymes. Additionally, the effect of these mutations on interaction with substrates and inhibitors was studied at the biochemical level using purified wild-type and mutant proteins.

**Results and Discussion**

**Effect of Amino Acid Substitutions in KPC-2 on MIC to Various Antibiotics.**

The effect of KPC mutations on resistance to various antibiotics was investigated. For these studies pUCP24 plasmids carrying wild-type and mutant *bla*KPC-2 genes as well as the pUCP24 vector were transformed into the efflux deficient strain of *P. aeruginosa* PAM1154. In this host, the effect of beta-lactamases on beta-lactam MIC is amplified due to the slowed uptake of beta-lactams across the low-permeability outer membrane without interference by efflux of either beta-lactams or BLIs. Consequently, this host allows detection of beta-lactamase activity (as an MIC increase) of low catalytic efficiency enzymes that rely heavily on low permeability of the outer membrane. Using the strain that lacks efflux pumps ensures no interference from efflux in interpreting results. We next evaluated the steady-state protein expression levels in bacterial cells. A western blotting experiment with whole cell lysates using anti-KPC-2 antibodies showed
no difference in protein expression levels in *P. aeruginosa* PAM1154 cells expressing both mutant proteins vs wild type KPC-2 (Supplementary Figure S1), suggesting no effect of mutations on protein stability. Previously, various amino acid substitutions at the position 179 of KPC-2 were shown to broadly reduce protein expression levels with the D179Y mutant demonstrating a several fold decrease compared to wild type protein (29). The observed discrepancy with our results could be attributed to the difference in either the expression vector or host bacteria.

MIC studies demonstrated that both mutations resulted in a 64-fold reduction of aztreonam and meropenem MICs: from 128 to 2 µg/ml and from 64 to 1 µg/ml, for aztreonam and meropenem, respectively. Of note, the MIC of the vector alone strain for these antibiotics was 0.125 µg/ml, indicating that the mutant KPC still afforded ca. 8-16-fold increase in aztreonam and meropenem MIC (Table 1). Cefepime MICs of the strains that carried mutant KPCs were reduced 4-fold, from 256 to 64 µg/ml, still affording 512-fold increase in MIC compared to the vector only strain. Piperacillin MICs were reduced 4-fold and 8-fold for the strains with D179Y and L169P mutants, respectively, from 128 to 32 µg/ml and 16 µg/ml, resulting in a 256-512-fold difference in MIC between the strains that carried KPC-mutations vs the vector alone cells. In contrast with other antibiotics, ceftazidime MICs were increased by both mutations: 16 and 8-fold increase for the D179Y and the L169P mutation, respectively. In general, our MIC results for D179Y are in good agreement with published data for multiple D179 substitutions reported in the KPC-2 enzyme: increased ceftazidime MICs, moderate decrease of MICs of other cephalosporins and a significant decrease in resistance to monobactams and carbapenems (22, 26, 29, 31, 32). Somewhat higher MICs of aztreonam and meropenem for D179Y reported in our study is most probably due to the host strain: *P. aeruginosa* as opposed to a more routinely used *E. coli* which
has a more permeable outer membrane with a consequent higher rate of beta-lactam uptake. Avibactam inhibited growth of all the strains with MIC of 128 µg/ml. MIC for vaborbactam was >256 µg/ml.

Effect of Amino Acid Substitutions in KPC-2 on the Potency of Avibactam and Vaborbactam to Enhance the Activity of Various Antibiotics.

The effect of mutations on BLI potency was investigated next. BLI potency was defined as PV$_{50}$ (PV, potentiation value) of antibiotic potentiation. PV$_{50}$ is the concentration of a BLI that is required to reduce the antibiotic MIC to the middle of the MIC range where the highest MIC is the MIC for a KPC-producing strain with no inhibitor added and the lowest MIC is the MIC for vector alone strain which corresponds to complete inhibition of KPC. As a given MIC is directly related to the beta-lactamase activity, PV$_{50}$ might be considered as a concentration of a BLI that is required to achieve half-effect of inhibition of beta-lactamase activity (in whole-cells) to hydrolyze an antibiotic of interest. The advantage of using PV$_{50}$ as a measure of BLI potency is that it does not depend on antibiotic MIC. However, the accurate determination of PV$_{50}$ requires the MIC range to be relatively wide. Based on these considerations, ceftazidime, cefepime and piperacillin were selected for checkerboard experiments.

Both avibactam and vaborbactam caused a dose-dependent decrease of ceftazidime, cefepime and piperacillin MICs in P. aeruginosa PAM1154 expressing wild-type KPC-2 and its mutants (Supplementary Table S1). Calculated PV$_{50}$ values are presented in Table 2.

The D179Y substitution in KPC-2 appeared to increase the avibactam PV$_{50}$ 4-, 16- and 32-fold for piperacillin, cefepime and ceftazidime, respectively, compared to the wild-type KPC-2.
The L169P mutation also appeared to decrease avibactam potency, albeit to a somewhat lesser degree: 4- and 8-fold increase in PV\textsubscript{50} for cefepime or piperacillin and ceftazidime, respectively. Of note, a decreased avibactam potency to reduce the MICs of the three tested antibiotics against the strains producing mutant proteins compared to the wild-type KPC-2 was observed, irrespective of the effect of mutations on antibiotics: reduction of cefepime and piperacillin MIC and an increase in ceftazidime MIC. This result is indicative of a possible direct effect of mutations on avibactam affinity for the KPC beta-lactamase. A recent study reported the impact of the D179Y substitution on activity of another BLI, clavulanic acid, which became more potent than avibactam in potentiating ceftazidime against the strain of E. coli with a cloned KPC-2::D179Y variant while being much less potent than avibactam against the wild type protein (31). However, since clavulanic acid is efficiently hydrolyzed by KPC (34), it is conceivable that a KPC variant that carries a D179Y substitution can lose the ability to inactivate clavulanic acid and as a result became more susceptible to inhibition.

Potentiation experiments with vaborbactam demonstrated that D179Y and L169P substitutions appear to affect PV\textsubscript{50} of vaborbactam to a much lesser degree compared to avibactam. None of the mutations decreased the potency of vaborbactam to enhance the activity of ceftazidime, cefepime or piperacillin more than two-fold (Table 2). While avibactam appeared to be 2-4-fold more potent than vaborbactam to potentiate antibiotics against the strain producing the wild-type KPC-2 (avibactam PV\textsubscript{50} of 0.125 µg/ml, 0.25 µg/ml and 0.25 µg/ml vs vaborbactam PV\textsubscript{50} of 0.5, 0.5 and 1 µg/ml for ceftazidime, cefepime and piperacillin, respectively), it became 4- and 2-fold less potent as a potentiator against the strain producing the D179Y and L169P variants, respectively. These results indicate that D179Y and L169P substitutions in the KPC-2 beta-lactamase had a lesser effect on interaction with vaborbactam.
Importantly, when antibiotic MICs against the strains producing KPC mutants were determined with BLIs at clinically relevant concentrations (4 µg/ml and 8 µg/ml for avibactam and vaborbactam, respectively), they were consistently lower for vaborbactam combinations compared to avibactam combinations. The highest difference, 8-fold, was for ceftazidime vs the strain producing D179Y variant: 8 µg/ml and 1 µg/ml for ceftazidime-avibactam and ceftazidime-vaborbactam, respectively.

The detection of the apparent impact of KPC mutations on interaction with avibactam (and to a much lesser degree with vaborbactam) became possible by generating complete concentration response curves of MIC of antibiotics vs BLI concentration for wild-type and mutant strains.

Comparing MICs of antibiotics against the wild-type and its mutants at a single inhibitor concentration might not allow discrimination between the effect of a mutation solely on antibiotic MIC (as was proposed based on earlier microbiological studies (20)) vs an additional possible direct effect on interaction with a BLI.

While the effect of mutations on BLI affinity is obviously an important factor that might contribute to resistance to a beta-lactam/BLI combination, their effect on antibiotic MIC is another factor potentially contributing to resistance. As an example, the MICs of ceftazidime and cefepime against the strain producing the D179Y variant are 256 µg/ml and 32 µg/ml, respectively, and \( PV_{50} \) for avibactam to potentiate ceftazidime or cefepime against this strain was found to be 4 µg/ml. At this concentration, the ceftazidime MIC is 8 µg/ml and the cefepime MIC is 4-fold lower or 2 µg/ml (Table 2). Of note, the group who first selected ceftazidime-avibactam-resistant mutants of KPC failed to get such selection in earlier studies with ceftaroline-avibactam (35). It will be very interesting to investigate whether or not it is purely due to a decrease in a
ceftaroline MIC (35). Irrespectively, the above result underscores the importance of a partner antibiotic when considering a combination with a beta-lactamase inhibitor.

In conclusion, our microbiological studies point to both the reduced affinity to avibactam (increased PV$_{50}$ for avibactam) and the specific role of ceftazidime in ceftazidime-avibactam resistance conferred by the D179Y and L169P substitutions. These data also indicate that the potency of vaborbactam is affected by KPC mutations to a lesser degree compared to that of avibactam.

**The Effect of Amino Acid Substitutions in KPC-2 on Beta-lactamase Activity**

Biochemical studies with the purified wild type KPC-2 and mutant proteins were carried out in an attempt to verify our microbiological results and potentially gain more mechanistic understanding on a biochemical level. First, we used a reporter substrate nitrocefin to determine kinetic parameters of KPC-2 variants. Purified D179Y and L169P proteins demonstrated nitrocefin $K_m$ values of 26±12 μM and 40±10 μM, which are similar to those of the wild type KPC-2 (Table 3). In contrast, D179Y and L169P mutants exhibited approximately 6000- and 300-fold reduction of nitrocefin $k_{cat}$ values, respectively. Of note, a similar reduction in $k_{cat}$ values of hydrolysis of another chromogenic cephalosporin, CENTA, by the D179Y variant was also reported in a recent study (31). This drop of hydrolytic activity for the mutant enzymes supports our microbiological observations that the strains expressing mutant KPC-2 proteins lost resistance to most antibiotics except ceftazidime and some cephalosporins. Next, we attempted to determine the kinetic parameters of ceftazidime hydrolysis for all three enzymes. Michaelis-Menten plots of reaction velocity vs substrate concentration are presented on Supplementary Figure S2. The wild type KPC-2 demonstrated no sign of saturation of reaction velocity with an increase of substrate concentration up to 2000 μM, indicating that the $K_m$ value is higher than that number.
and preventing separate calculations of $K_m$ and $k_{cat}$ values. This finding is in good agreement with the previously reported data (36, 37). In contrast, the rate of ceftazidime hydrolysis for the D179Y mutant remained constant in a wide range of substrate concentrations suggesting that the $K_m$ value is very low (below the detection limit); thus, this type of kinetic behavior allows only calculating the $k_{cat}$ value (Supplementary Figure S2, Table 3). Accordingly, for the wild-type KPC-2 and the D179Y mutant, we used a different method that allows calculation of $k_{cat}/K_m$ catalytic ratio by analyzing complete ceftazidime cleavage profiles. For KPC-2 wild type, the $k_{cat}/K_m$ value was $0.00087 \pm 0.00011 \mu M^{-1}s^{-1}$, while for D179Y this value was ten-fold higher - $0.0088 \pm 0.0009 \mu M^{-1}s^{-1}$.

Unlike the wild-type KPC-2 and the D179Y mutant, the L169P mutant demonstrated a typical Michaelis-Menten plot with a $K_m$ value of 27±6 μM and $k_{cat}$ of 0.093±0.009 s$^{-1}$ (Supplementary Figure S2, Table 3); hence the $k_{cat}/K_m$ value for the L169P mutant was obtained from separate $K_m$ and $k_{cat}$ numbers and was $0.0036 \pm 0.0009 \mu M^{-1}s^{-1}$.

The observed increase in $k_{cat}/K_m$ for both mutants may explain the higher ceftazidime MIC values observed for the cloned mutant KPC genes (Table 1). The predicted ceftazidime $K_m$ of the D179Y mutant (calculated by division of $k_{cat}$ by $k_{cat}/K_m$) is around 1 μM, which is more than 2000-fold lower than that of the wild type KPC-2. Consistent with our findings, a quick burst of ceftazidime hydrolysis has been reported for the KPC-2 D179N mutant when studied using the stop flow kinetic technique; the authors believe that this burst of ceftazidime hydrolysis was due to the tight ceftazidime binding to the mutant protein (29). It is also conceivable that the high increase in affinity to ceftazidime for the D179Y mutant could prevent avibactam from efficiently binding to the enzyme and thus contribute to the resistance mechanism.
The Effect of Amino Acid Substitutions in KPC-2 on Inhibition by Avibactam and Vaborbactam

We next attempted to determine the effect of mutations on interactions with avibactam and vaborbactam using nitrocefin as a substrate. It has been reported that both BLIs behave as slow tight binding inhibitors of KPC-2, which is presented by the following kinetic scheme (12, 38).

\[ \text{E + I} \leftrightarrow \text{EI} \leftrightarrow \text{EI}^* \], where \( K = k_1 / k_2 \) and \( K_d = K \times k_2 / (k_2 + k_2) \)

This type of inhibition is manifested by a progressive inactivation phenomenon when studied using the “reporter substrate” method (Supplementary Figure S3). Consequently, the affinity of such inhibitors to β-lactamases is quantitatively characterized by the inactivation constant \( k_2/K (k_{inact}) \) and dissociation constant \( k_2 \) with overall BLI affinity characterized by \( K_d \) values. We have recently reported vaborbactam inhibition parameters for KPC-2 (12) and here we determined the avibactam inhibition parameters (Table 4). Our data are very similar to the previously reported results (38). Avibactam and vaborbactam \( K_d \)s for KPC-2 were very similar, 0.014 μM and 0.008 μM, respectively (Table 4). Unexpectedly, the KPC-2 L169P mutant protein produced linear enzyme inactivation profiles with both inhibitors that are typical of “fast on – fast off” boronic BLIs (Supplementary Figure S4)). This finding precluded calculation of \( k_2/K \) and \( k_2 \) values for this mutant. Hence, avibactam and vaborbactam steady-state \( K_i \) values.
were determined for the L169P mutant using the method that has been previously utilized for “fast on – fast off” boronic BLIs with nitrocefin as a substrate (39, 40). Kᵢ values were found to be $0.89 \pm 0.03 \mu M$ and $0.19 \pm 0.02 \mu M$, for avibactam and vaborbactam, respectively, or ~4.5-fold higher for avibactam compared to vaborbactam (Table 4).

The apparent difference in inhibition kinetics observed for wild-type KPC-2 and its L169P derivative precluded us from directly comparing inhibition constants observed for each inhibitor for the wild-type vs mutant KPC-2. However, while both inhibitors apparently had a similar affinity for the wild-type KPC-2, the affinity of avibactam for the mutant L169P protein was ~4.5-fold lower as compared to vaborbactam (Table 4). This result indicated that the L169P amino acid substitution in KPC-2 affected avibactam more strongly than vaborbactam, which is similar to the results from microbiological experiments (2-fold to 8-fold stronger effect of the L169P substitution on avibactam PV₅₀ vs vaborbactam PV₅₀ depending on the antibiotic). The logical conclusion is that the L169P substitution does affect the affinity of avibactam for the KPC-2 beta-lactamase though the exact magnitude of the effect remains to be determined in future experiments. This potential reduced affinity for avibactam can contribute to ceftazidime-avibactam resistance in addition to the previously described increase in ceftazidime hydrolytic activity observed for this mutant.

The results of evaluation of the impact of avibactam and vaborbactam on nitrocefin hydrolysis mediated by the D179Y mutant were unexpected. Avibactam did not inhibit this hydrolysis even when used at a very high concentration of 2560 µM. Vaborbactam demonstrated some inactivation effect at 2560 µM, but a rough estimation of the $k_2/K$ value resulted in an almost 10000-fold decrease in inactivation efficiency compared to the wild type KPC-2 (Figure S4). Both avibactam and vaborbactam findings are at odds with our microbiological data that
indicate that both inhibitors are capable of enhancing the activity of various antibiotics against the
strain producing the D179Y mutation with potency that is only two-fold or 8 to 32-fold lower for
vaborbactam and avibactam, respectively, compared to that observed against the strain producing
the wild-type KPC-2 beta-lactamase (Table 2). We believe that the abnormally high resistance to
inhibition observed in the nitrocefin (and possibly, CENTA) hydrolysis assay with the D179Y
mutant might be an artifact attributed to the extremely low rate of nitrocefin hydrolysis.

Owing to the ability of the D179Y variant to hydrolyze ceftazidime, we attempted to
compare binding affinities of avibactam and vaborbactam for the wild-type KPC-2 and the
D179Y mutant by measuring the IC$_{50}$ of inhibition of ceftazidime hydrolysis. To account for the
different ceftazidime $K_m$ values for these proteins, ceftazidime was used at 100 μM and at 10 μM
for the wild-type KPC-2 and the D179Y mutant, respectively. The results are presented in Table
5. We first compared the potency of avibactam and vaborbactam against the same KPC variant.
For the wild-type KPC-2, the IC$_{50}$ value of avibactam (0.47±0.02 μM) was ca. two-fold lower
compared to that of vaborbactam (0.94±0.02 μM), somewhat reminiscent of the 2-4-fold lower
PV$_{50}$ values for avibactam obtained in microbiological experiments (Table 2). In contrast, for the
D179Y mutant protein, the IC$_{50}$ value of avibactam (8.9±0.9 μM) was ca. 4.5-fold higher
(reduced potency) compared to that of vaborbactam (1.9±0.3 μM) (Table 5), again somewhat
similar to the 4-fold lower potency (higher PV$_{50}$ values) of avibactam compared to vaborbactam
for the strain producing KPC-2 with the D179Y substitution.

We are precluded from establishing whether the D179Y substitution affected the affinity
of BLIs for KPC-2, let alone to accurately estimating the magnitude of its potential effect, by
comparing IC$_{50}$ values for the wild-type vs. mutant KPC-2 that were determined at different
substrate saturation conditions. However, it is possible to compare avibactam and vaborbactam
based on the impact caused by the mutation by comparing the changes in IC₅₀. The avibactam IC₅₀ value was increased almost 20-fold, from 0.47±0.02 µM for wild type KPC-2 to 8.9±0.9 µM for D179Y (Table 5). At the same time the vaborbactam IC₅₀ was increased only two-fold – from 0.94±0.02 µM to 1.9±0.3 µM, indicating the different impact of D179Y on two different BLIs. Based on this result we conclude that the D179Y substitution has a direct effect on the affinity of avibactam and vaborbactam but the effect on avibactam is stronger than that on vaborbactam. As was the case with L169P, the determination of the exact magnitude of the effect on inhibition parameters will probably require different experimental technique.

**Conclusions**

In conclusion, our microbiological studies pointed both to the specific role of ceftazidime, possibly due to increased efficiency of ceftazidime hydrolysis, and the reduced affinity to avibactam, in ceftazidime-avibactam resistance conferred by the D179Y and L169P substitutions. These studies were in a good agreement with biochemical experiments. Purified KPC-2 D179Y and L169P enzymes demonstrated a higher catalytic ratio $k_{cat}/K_m$ of ceftazidime hydrolysis compared to the wild type protein as well as significantly reduced ceftazidime $K_m$ values. It is plausible that the former may explain the increased ceftazidime resistance associated with both mutations while the latter is responsible for the observed reduction of avibactam potency to enhance activity of ceftazidime in microbiological experiments. In addition, the D179Y and L169P substitutions appeared to have a direct effect on avibactam binding affinity for KPC-2; this may explain the negative impact of both mutations on potentiation of other antibiotics. These mutations had a lesser effect on both the enzyme inhibition and antibiotic potentiation activity of vaborbactam. In addition, KPC mutations that
confer resistance to ceftazidime-avibactam resulted in the significantly reduced resistance to meropenem. This makes the meropenem-vaborbactam combination a valuable agent in managing infections due to KPC-producing carbapenem resistant Enterobacteriaceae.

Materials and Methods

Generation of KPC-2 mutants

Mutations in the blaKPC-2 gene cloned in either pUCP24 or pET28a plasmids were introduced using the QuickChange Lightning Site-Directed Mutagenesis Kit (ThermoFisher Scientific, USA).

Susceptibility testing

For microbiological studies, wild type blaKPC-2 and its mutant variants were cloned into the shuttle vector pUCP24. Resulting plasmids were transformed in the efflux-deficient strain of P. aeruginosa PAM1154 using selection on 15 µg/mL of gentamicin. Using a strain of P. aeruginosa as opposed to E. coli as a host for various cloned genes was based on the following consideration. As well-documented elsewhere (41), P. aeruginosa has a low-permeability outer membrane; consequently, the effect of beta-lactamases on MIC is amplified in P. aeruginosa compared to E. coli due to the slowed uptake of beta-lactams. Thus, this host is appropriate to detect a beta-lactamase activity (as an MIC increase) of low catalytic efficiency enzymes that rely heavily on low permeability of the outer membrane. PAM1154 lacks major efflux pumps, so that efflux does not interfere with microbiological potency of both beta-lactams and beta-lactamase inhibitors. MIC values were determined using Clinical and Laboratory Standards Institute (CLSI)
broth microdilution method as described in CLSI document M07-A11 (42). Potentiation of antibiotic activity by various BLIs in bacterial strains carrying wild type (WT) and mutants KPC-2 genes were performed using standard checkerboard methodology (43). BLI potency was defined as $PV_{50}$ (potentiation value). $PV_{50}$ is a minimal concentration of a BLI that is required to reduce antibiotic MIC to the middle of the MIC range ($E_{50}$) where the highest MIC is the MIC for a KPC-producing strain with no inhibitor added and the lowest MIC is the MIC for the vector only strain, corresponding to the complete inhibition of KPC. $E_{50}$ is calculated as the square root of the product of the antibiotic MIC values for the KPC-producing and the vector only strain.

The results of checkerboard experiments were highly reproducible.

**Evaluation of KPC-2 mutant proteins expression level in *P.a.* PAM1154 strain**

Bacterial cells carrying plasmids expressing KPC-2 WT and mutants were grown in liquid media to an OD$_{600}$=0.7-0.9 and diluted to final OD$_{600}$=0.5. 500 µl of cell culture was spun down and the resulting pellet was resuspended in 500 µL of gel loading buffer. 20 µL of cell lysate were loaded onto 8-16% SDS-PAGE. After transfer, the membrane was probed with custom produced rat polyclonal anti-KPC-2 antibodies and subsequently treated with secondary goat anti-rat HRP-conjugated antibodies. Anti-RNA polymerase β-subunit monoclonal antibodies (Abcam, Burlingame, CA USA #ab12087) were used as a loading control.

**Purification of the wild type KPC-2 and D179Y mutant proteins**

KPC-2 gene coding sequence was cloned into a pET28a vector that produced the construct with periplasmic KPC-2 secretion and 6xHis-tag on its C-terminus. The recombinant plasmids were transformed into the BL21(DE3) pLys strain. Protein expression was induced by 0.2 mM IPTG for 3 hours at 37°C. The cell pellet was lysed in ice-cold 50 mM TrisHCl pH 8.0, 500 mM
sucrose, 1 mM EDTA with six cycles of 15 seconds vortexing and 5 min incubation on ice. After centrifugation, the supernatant was adjusted with MgCl₂ and imidazole to 5 mM and 4 mM, respectively. The lysate was loaded onto 1 mL column with HisPur Cobalt resin (ThermoFisher Scientific, USA) pre-equilibrated with 50 mM Na-phosphate pH 7.4, 300 mM NaCl, 4 mM imidazole buffer. The column was washed with 40 mL of the same buffer and consequently the His-tag protein was eluted with a linear gradient of 4 mM to 70 mM imidazole in 50 mM Na-phosphate pH 7.4, 300 mM NaCl buffer. All fractions were analyzed by 8-16% SDS-PAGE. Fractions containing target protein were pooled, concentrated and dialyzed against 50 mM Na-phosphate pH 7.0.

**Purification of KPC-2 L169P mutant protein**

Protein expression was induced by 0.2 mM IPTG overnight at 18°C. The cell pellet was lysed in ice-cold 50 mM Na-phosphate pH 7.4, 300 mM NaCl, 4 mM imidazole with seven cycles of 1 min sonication on ice. After centrifugation, the lysate was loaded onto 1 mL column with HisPur Cobalt resin pre-equilibrated with 50 mM Na-phosphate pH 7.4, 300 mM NaCl, 4 mM imidazole buffer. The column was washed with 40 mL of the same buffer and consequently the His-tag protein was eluted with a linear gradient of 4 mM to 70 mM imidazole in 50 mM Na-phosphate pH 7.4, 300 mM NaCl buffer. All fractions were analyzed by 8-16% SDS-PAGE. Fractions containing target protein were pooled, concentrated and dialyzed against 50 mM Na-phosphate pH 7.0.

**Determination of $K_m$ and $k_{cat}$ values for nitrocefin and ceftazidime cleavage by KPC-2 WT and mutant proteins**

Enzymes were mixed with various concentrations of nitrocefin in 50 mM sodium phosphate pH 7.0, 0.1 mg/mL bovine serum albumin (buffer A) and substrate cleavage was monitored at 490
nm every 10 seconds for 10 minutes at 37°C on a SpectraMax plate reader (Molecular Devices, San Jose, CA, USA). Initial rates of nitrocefin cleavage were calculated and used to obtain $K_m$ and $k_{cat}$ values with Prism software (GraphPad, San Diego, CA USA). For ceftazidime kinetic parameters calculation, enzymes were mixed with various concentrations of substrate in buffer A, transferred in either 1 mm or 10 mm light path quartz cuvette and substrate cleavage was monitored at 260 nm every 30 seconds for 10 min at room temperature. Initial rates of ceftazidime cleavage were calculated and used to obtain $K_m$ and $k_{cat}$ values with Prism software.

**Determination of $k_{cat}/K_m$ ratio for ceftazidime cleavage by purified enzymes**

Wild-type KPC-2 enzyme was mixed with 250 µM ceftazidime in buffer A and the reaction mix was transferred to a 1 mm light path quartz cuvette. For D179Y and L169P mutants, enzyme was mixed with 2.5 µM ceftazidime in buffer A and the reaction mix was transferred to a 10 mm light path quartz cuvette. Substrate cleavage was monitored at 260 nm every 30 seconds at room temperature using a SpectraMax plate reader. The reaction was monitored until $OD_{260}$ values reached a plateau. Resulting $OD_{260}$ vs time reaction profiles were fitted to the following equation using Prism software:

$$A_t = A_o + (A_0 - A_o) \cdot e^{-kt},$$

where $A_t$ – absorbance at time $t$, $A_0$ – initial absorbance, $A_o$ – final absorbance.

In this equation $k = k_{cat}/K_m \cdot [E]$ that allows to calculate $k_{cat}/K_m$ knowing enzyme concentration (44).

**Determination of avibactam $k_2/K$ inactivation constant for KPC-2 enzyme.**
Inactivation kinetic parameters were determined by reporter substrate method for slow tight binding inhibitor kinetic scheme (45). Protein was quickly mixed with 100 μM nitrocefin and various concentrations of BLI in reaction buffer and absorbance at 490 nm was measured immediately every two seconds for 600 sec on SpectraMax plate reader (Molecular Devices, San Jose, CA, USA) at 37°C. Resulting progression curves of OD490 vs time at various BLI concentrations were imported into Prism software (GraphPad, San Diego, CA, USA) and pseudo first-order rate constants kobs were calculated using the following equation:

\[ P = V_s \times (1 - e^{-k_{obs} \times t}) / k_{obs}, \]

where \( V_s \) - enzyme nitrocefin cleavage rate in the absence of BLI, \( k_{obs} \) values calculated at various vaborbactam concentrations were fitted in the following equation:

\[ k_{obs} = k_{-2} + k_{2}/K \times [I] / (1+[NCF]/K_{m}(NCF)), \]

where \( k_{-2} \) - inactivation constant

\( [I] \) – inhibitor concentration

\( [NCF] \) – nitrocefin concentration

\( K_{m}(NCF) \) - Michaelis constant of NCF for KPC-2

**Determination of \( k_{-2} \) rates of KPC-2 enzyme activity recovery after inhibition by avibactam.**

Purified KPC-2 at 1 μM concentration in buffer A was mixed with BLIs at 8-fold higher concentration than its stoichiometry ratio (determined in preliminary stoichiometry experiments). After 30 min incubation at 37°C, the reaction mixture was diluted 10000-fold in buffer A and 100 μL of diluted enzyme was mixed with 100 μL of 400 μM nitrocefin in reaction buffer. Absorbance at 490 nm was recorded every minute during 4h at 37°C. Resulting reaction profiles were fitted into the following equation using Graph Pad Prizm software to obtain \( k_{-2} \) values:

\[ P = V_s \times t + (V_o - V_s) \times (1 - e^{-k_{-2} \times t}) / k_{-2}, \]

where \( V_s \) – uninhibited enzyme velocity, measured in the reaction
with enzyme and no inhibitor, \( V_o \) – completely inhibited enzyme velocity, measured in the reaction with no enzyme and nitrocefin only.

### Determination of \( K_i \) values of \( \beta \)-lactamase inhibition by BLIs with nitrocefin as a substrate

Enzymes were mixed with BLIs at concentrations varying from 160 to 0.0027 µM in buffer A, incubated for 10 minutes at 37°C and NCF substrate was subsequently added. Substrate cleavage profiles were recorded at 490 nm at 37°C every 10 seconds for 10 min. \( K_i \) values were calculated by method of Waley SG (46). The reaction profiles with and without inhibitor are compared and several values of the time difference \((t-t_c)\) (where \( t \) and \( t_c \) are times to reach given substrate concentration in the reaction with and without inhibitor, respectively) for equal values of substrate concentration \( s \) are obtained. Then \((t-t_c)\) is plotted against \( \ln(s_0/s) \) \((s_0 \) is initial substrate concentration) and the slope is measured. \( K_i \) is calculated using the following equation:

\[
K_{\text{app}} = [I] \times \frac{K_m}{V_{\text{max}}} \times \frac{1}{\text{Slope}}
\]

where \([I]\) – inhibitor concentration, \( K_m \) and \( V_{\text{max}} \) – Michaelis-Menten constants of NCF for KPC-2 and mutant proteins.

### Determination of IC\(_{50}\) values of inhibition of KPC-2 variants by BLIs with ceftazidime as a substrate

Enzymes were mixed with BLIs at concentrations varying from 160 to 0.0027 µM in buffer A, incubated for 10 minutes and 100 µM (for wild type KPC-2) or 10 µM (for D179Y mutant) CFTZ was subsequently added. Substrate cleavage profiles were recorded at 260 nm every 10 seconds for 10 min. Initial rates of reaction were calculated and exported to Prizm software to calculate IC\(_{50}\) values using “dose-response – inhibition, variable slope (four parameters)” equation.
Statistical analysis. All kinetic results are presented as average ± standard deviation of minimum three replicates.

Funding Information.

This project was funded in part by Federal funds from the Department of Health and Human Services, Office of the Assistant Secretary for Preparedness and Response, Biomedical Advanced Research and Development Authority (BARDA), under contract HHSO100201400002C with Rempex Pharmaceuticals, a wholly-owned subsidiary of The Medicines Company and agreement HHSO100201600026C with The Medicines Company.

Acknowledgments

The authors are grateful to Scott Hecker, Sandra McCurdy and Mark Redell for the critical reading of the manuscript and useful comments.

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### Table 1. MIC values (μg/ml) for the *P. aeruginosa* PAM1154 carrying plasmids with the wild type KPC-2 or the corresponding mutant proteins

| Strain      | Plasmid                  | Aztreonam | Meropenem | Ceftazidime | Cefepime | Piperacillin | Avibactam |
|-------------|--------------------------|-----------|-----------|-------------|----------|--------------|-----------|
| PAM4175     | pUCP24                   | 0.125     | 0.125     | 0.25        | 0.125    | 0.06         | 128       |
| PAM4135     | pUCP24-KPC-2             | 128       | 64        | 32          | 256      | 128          | 128       |
| PAM4639     | pUCP24-KPC-2::D179Y      | 2         | 1         | 512         | 64       | 32           | 128       |
| PAM4751     | pUCP24-KPC-2::L169P      | 2         | 1         | 256         | 64       | 16           | 128       |

Vaborbactam MIC values are >256 μg/ml for all the strains.

### Table 2. MIC values (μg/ml) of ceftazidime, cefepime and piperacillin alone or in combination with BLIs for the *P. aeruginosa* PAM1154 containing plasmids with the wild type KPC-2 or the corresponding mutant proteins

| Strain      | KPC variant          | Antibiotic | No BLI | w/Avibactam at 4 µg/ml | w/Vaborbactam at 8 µg/ml | PV\(_{50}\) (µg/ml) |
|-------------|----------------------|------------|--------|------------------------|--------------------------|-------------------|
| PAM4175     | pUCP24 (vector)      | ceftazidime| 0.25   | 0.25                   | 0.25                     | Avibactam         |
| PAM4135     | KPC-2                | ceftazidime| 16     | 0.25                   | 0.25                     | 0.125             |
| PAM4639     | KPC-2::D179Y         | ceftazidime| 256    | 8                      | 1                        | 4                 |
| PAM4751     | KPC-2::L169P         | ceftazidime| 128    | 2                      | 0.5                      | 2                 |
| PAM4175     | pUCP24 (vector)      | cefepime   | 0.125  | 0.125                  | 0.125                    | Avibactam         |
| PAM4135     | KPC-2                | cefepime   | 64     | 0.125                  | 0.125                    | 0.25              |
| PAM4639     | KPC-2::D179Y         | cefepime   | 32     | 2                      | 0.25                     | 4                 |
| PAM4751     | KPC-2::L169P         | cefepime   | 32     | 0.5                    | 0.125                    | 1                 |
| PAM4175     | pUCP24 (vector)      | piperacillin| 0.125  | 0.125                  | 0.125                    | Vaborbactam       |
| PAM4135     | KPC-2                | piperacillin| 128    | 0.5                    | 0.25                     | 1                 |
| PAM4639     | KPC-2::D179Y         | piperacillin| 32     | 1                      | 0.25                     | 1                 |
| PAM4751     | KPC-2::L169P         | piperacillin| 16     | 0.5                    | 0.25                     | 1                 |

PV\(_{50}\) is a minimal concentration of a BLI that is required to reduce antibiotic MIC to the middle of the MIC range (E\(_{50}\)) where the highest MIC is the MIC for a KPC-producing strain with no inhibitor added and the lowest MIC is the MIC for vector only strain, corresponding to the complete inhibition of KPC. E\(_{50}\) is calculated as the square root of the product of the antibiotic MIC values for the KPC-producing and the vector only strain.
Table 3. Kinetic parameters of nitrocefin and ceftazidime hydrolysis by KPC-2 and mutant proteins

| Enzyme       | $K_{\text{m}}$ μM | $k_{\text{cat}}$ s$^{-1}$ | $k_{\text{cat}}/K_{\text{m}}$ s$^{-1}$ μM$^{-1}$ | $K_{\text{m}}$ μM | $k_{\text{cat}}$ s$^{-1}$ | $k_{\text{cat}}/K_{\text{m}}$ s$^{-1}$ μM$^{-1}$ |
|--------------|-------------------|-----------------------------|-------------------------------------------------|-------------------|-----------------------------|-------------------------------------------------|
| KPC-2        | 36 ± 5            | 132 ± 13                    | 3.7 ± 0.2                                        | >2000             | >2.6                        | 0.00087 ± 0.00011*                                |
| KPC-2 D179Y  | 26 ± 12           | 0.023 ± 0.002               | 0.0010 ± 0.0005                                  | <5                | 0.0088 ± 0.0003             | 0.0088 ± 0.0009*                                  |
| KPC-2 L169P  | 40 ± 10           | 0.47 ± 0.04                 | 0.012 ± 0.002                                    | 27 ± 6            | 0.093 ± 0.009               | 0.0036 ± 0.0009                                   |

$k_{\text{cat}}/K_{\text{m}}$ values were calculated by method described in (44).

Table 4. Kinetic parameters of vaborbactam and avibactam inhibition of NCF hydrolysis by KPC-2 and L169P mutant

| Enzyme       | $k_2/K_1$ M$^{-1}$ s$^{-1}$ | $k_2, s^{-1}$ | $K_a$ μM | $K_i$ μM |
|--------------|------------------------------|---------------|----------|----------|
| KPC-2        | $2.3 \pm 0.2 \times 10^4$   | $3.3 \pm 0.1 \times 10^{-4}$ | 0.014 ± 0.001 | 0.001     |
| KPC-2 L169P  | 0.89 ± 0.03                  |               |          |          |

Table 5. IC$_{50}$ values of vaborbactam and avibactam inhibition of ceftazidime hydrolysis by KPC-2 and D179Y mutant

| Enzyme       | Avibactam IC$_{50}$ μM | Vaborbactam IC$_{50}$ μM |
|--------------|-------------------------|---------------------------|
| KPC-2        | 0.47 ± 0.02             | 0.94 ± 0.02               |
| KPC-2 D179Y  | 8.9 ± 0.9               | 1.9 ± 0.3                 |

$100 \mu$M and 10 μM of ceftazidime was used to determine IC$_{50}$ values for KPC-2 and its D179Y mutant, respectively.
