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Vaborbactam (VAB) is not affected by KPC-2 and KPC-3 Variants Containing Asp179Tyr Amino Acid Substitution that are Resistant to Ceftazidime (CAZ) Potentiation with Avibactam

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Abstract

Background: Single-step resistance development studies using CAZ-AVI as a selective agent have identified several mutations in the blaKPC-3 gene that conferred resistance to this combination. One of these mutations, D179Y, have recently appeared in KPC-3 producing clinical isolates recovered from patients treated with CAZ-AVI. The objective of these studies was to evaluate the effect of D179Y mutation on VAB interaction with KPC-2 and KPC-3 enzymes.

Methods: D179Y was introduced in blaKPC by site-directed mutagenesis (KPC-D179Y). Susceptibility of the strains of P. aeruginosa (PAM1154 background, lacks major efflux pumps) producing either wild type (wt) KPC-2/KPC-3 or KPC-D179Y, to various antibiotics and BLI combinations were tested. Kinetic parameters (kcat, Km) of purified wt KPCs and KPC-D179Y and Ki for VAB and AVI were determined spectrophotometrically using nitrocefin (NCF) and CAZ as substrates.

Results: D179Y mutation in KPC-3 or KPC-2 resulted in the 4-16-fold increase in CAZ MIC and in the concomitant 32-64-fold decrease in aztreonam and meropenem MIC. The D179Y mutants in either KPC-2 or KPC-3 were also resistant to potentiation of CAZ by AVI compared to the wt proteins: AVI at 4 µg/ml reduced CAZ MIC from 32-128 µg/ml to 0.5-1 µg/ml against the strains producing wt KPC-2 or KPC-3, respectively (64-128-fold effect); against KPC-D179Y mutants CAZ MIC were reduced only 16-fold, from 512 µg/ml to 32 µg/ml. Notably, the D179Y mutation had much less effect on potentiation of CAZ by VAB: VAB at 4 µg/ml reduced CAZ MIC to 1 µg/ml against the KPC-2 mutant (256-effect, the same as against the wt KPC-2) and to 8 µg/ml against the KPC-3 mutant (64-fold effect, only 4-fold lower compared to the wt KPC-3). Studies with purified proteins using NCF as a substrate indicated that there were no changes in kinetics of inhibition of D179Y mutants by either AVI or VAB compared to the wt proteins. When CAZ was used as a substrate, KPC-2-D179Y mutant demonstrated ~10-fold increase in the catalytic efficiency and >2000-fold decrease in CAZ Km, from >2000 µM to 1 µM. The AVI Ki was increased 30-fold for D179Y compared to the wt KPC-2. Only a 3-fold increase in Ki was recorded for VAB.

Conclusions: Both microbiological and biochemical data demonstrate that the effect of D179Y mutation is specific for KPC-mediated CAZ hydrolysis and its inhibition by AVI. These studies show retention of VAB inhibitory activity of KPC enzymes with mutations shown to reduce the potency of inhibition by AVI, and are consistent with a distinct mode for VAB binding to the active site compared to AVI.

Introduction

- Single-step resistance development studies using ceftazidime-avibactam as a selective agent have identified several mutations in the *bla*$_{KPC-3}$ gene that conferred resistance to this combination (1).
- One of these mutations, D179Y, has recently been detected in KPC-3 producing clinical isolates of Enterobacteriaceae recovered from patients treated with ceftazidime-avibactam (2).
- Vaborbactam (formerly known as RPX7009) is a novel beta-lactamase inhibitor with potent activity against KPC. It is based on cyclic boronic acid pharmacophore and thus is structurally different from avibactam (3). Recent studies have also shown that its mode of binding to beta-lactamases is different from avibactam (4).
- Given the clinical significance associated with resistance to ceftazidime-avibactam following treatment, additional treatments appear to be required (5).
- The objective of these studies was to evaluate the effect of D179Y mutation on VAB inhibition of interaction with KPC-2 and KPC-3 enzymes.

Methods

Generation of KPC-2 and KPC-3 mutants

Mutations in the *bla*$_{KPC-2}$ or *bla*$_{KPC-3}$ genes cloned in either pUCP24 or pET28a plasmids were introduced using "QuickChange Lightning Site-Directed Mutagenesis Kit" (Agilent Technologies).

Susceptibility testing

For microbiological studies wild type *bla*$_{KPC-2}$ or *bla*$_{KPC-3}$ and their mutant variants were cloned into the shuttle vector pUCP24. Resulting plasmids were transformed in the strain PAM1154 of *P. aeruginosa* that lacks major efflux pumps using selection on 15 µg/ml of gentamicin. MICs were determined using Clinical and Laboratory Standards Institute (CLSI) broth microdilution method as described in CLSI document M07-A17 (6). Potentiation of antibiotic activity by various BLIs in bacterial strains carrying wild type and mutants KPC-2 genes were performed using standard checkerboard methodology (7).

Evaluation of KPC-2 mutant proteins expression level in PAM1154 strain

Bacterial cells carrying plasmids expressing KPC-2 wt and D179Y mutant were grown in liquid media to an OD600=0.7-0.9 and diluted to final OD600=0.5. 500 ml of cell culture were spun down and the resulting pellet was resuspended in 500 ml of gel loading buffer. 20 ml of cell lysate were loaded on 8-16% SDS-PAGE. After transfer membrane was probed with custom produced rat anti-KPC-2 antibodies and subsequently treated with secondary goat anti-rat HRP-conjugated antibodies. Anti-RNA polymerase beta-subunit monoclonal antibodies (Abcam, #ab12087) were used as a loading control.

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

KPC-2 gene coding sequence with its SD box 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. Cell pellet was lysed in ice-cold 50 mM TrisHCl pH8.0, 500 mM sucrose, 1 mM EDTA with six cycles of 15 seconds vortexing. After centrifugation supernatant was adjusted with $MgCl_2$ and imidazole to 2 mM and 5 mM, respectively. Lysate was loaded by gravity flow onto 1ml column with HisPur Cobalt Resin pre-equilibrated with 50 mM Na-phosphate pH7.4, 300 mM NaCl, 5 mM imidazole buffer. Column was washed with 40 ml of the same buffer and consequently His-tag protein was eluted with 50 mM Na-phosphate pH7.4, 300 mM NaCl, 70 mM imidazole buffer. All fractions were analyzed by 8-16% SDS-PAGE. Fractions containing the target protein were pooled, concentrated and dialyzed against 50 mM Na-phosphate pH7.0.

Determination of K_m and k_{cat} values for nitrocefin and ceftazidime cleavage by KPC-2 wt and KPC-2-D179Y proteins

Enzymes were mixed with various concentrations of nitrocefin (NCF) in 50 mM sodium phosphate pH7.0, 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 SpectraMax plate reader. Initial rates of NCF cleavage were calculated and used to obtain K_m and k_{cat} values with Prism software ("GraphPad"). 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 1h at room temperature. Initial rates of ceftazidime cleavage were calculated and used to obtain K_m and k_{cat} values with Prism software ("GraphPad").

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 1mm light path quartz cuvette. D179Y mutant, enzyme was mixed with 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 for 2h at room temperature using SpectraMax plate reader. The reaction was continued until OD_{260} values reached a plateau. Resulting OD_{260} vs time reaction profiles were fitted to the following equation using Prism software ("GraphPad")

$A_t=A_\infty+(A_0-A_\infty)*e^{-k*t}$, where A_t – absorbance at time t, A_0 – initial absorbance, A_∞ – final absorbance.

In this equation $k=k_{cat}/K_m*[E]$ that allows to calculate k_{cat}/K_m knowing enzyme concentration [E] (8).

Determination of K_i values of beta-lactamase inhibition by BLIs with nitrocefin or ceftazidime as substrates

Enzymes were mixed with BLIs at concentrations varying from 160 to 0.0027 µM in buffer A and incubated for 10 minutes at 37° C. 50 µM NCF (pre-warmed at 37° C for 10 minutes) was added and substrate cleavage profiles were recorded at 490 nm every 10 seconds for 10 minutes. For ceftazidime substrate enzymes were mixed with various concentrations of BLIs in buffer A and either preincubated for 10 minutes at room temperature before addition of 100 µM ceftazidime or substrate was added immediately after that. Reaction mixture was transferred to 10 mm cuvette and substrate cleavage profiles were recorded at 260 nm every 30 seconds for 1 hour. For both substrates K_i values were calculated by method of Waley SG (9).

Determination of k_2/K inactivation constant for KPC-2 and KPC-2-D179Y proteins

Inactivation kinetic parameters were determined by reporter substrate method (10) for slow-tight binding inhibitors behaving according to the kinetic scheme below.

$$E + I \overset{K}{\leftrightarrow} EI \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} EI^*$$

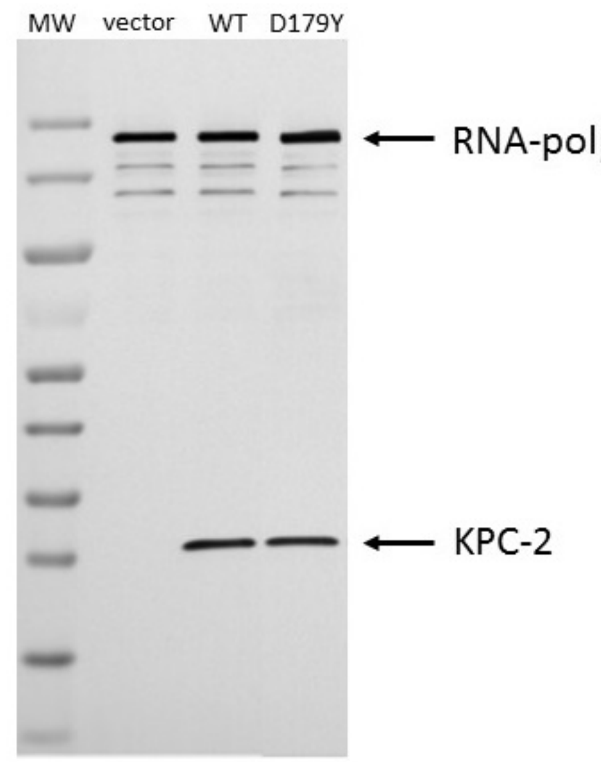
Enzymes (KPC-2, 0.8 nM; KPC-2-D179Y, 0.5 µM) were quickly mixed with 100 µM of NCF and various concentrations of BLIs in the reaction buffer and absorbance at 490 nm was measured immediately every two seconds for 300 seconds on a SpectraMax plate reader. The resulting progression curves of OD490 vs. time at various vaborbactam concentrations were imported into Prism software ("GraphPad") and pseudo first-order rate constants K_{obs} were calculated using the following equation: $P=V_0*(1-e^{-kobs*t})/k_{obs}$, where V_0 = uninhibited enzyme rate. K_{obs} values were fitted in the following equation: $k_{obs}=k_{-2}+k_2/K*[I]/(1+[NCF]/K_m(NCF))$, where k_2/K = inactivation constant, [I] = inhibitor concentration, [NCF] = nitrocefin concentration, K_m(NCF) = Michaelis constant of NCF for KPC-2 wt and mutant enzymes.

Determination of K_{off} rates of enzyme activity recovery after inhibition by BLIs.

Purified enzyme at 1 µM concentration in reaction buffer was mixed with vaborbactam at 8-fold higher concentration than its stoichiometric ratio. After 30 minutes 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 NCF. Absorbance at 490 nm was recorded for 4 hours each 1 minute. Reaction profiles were fitted into the following equation using "Prizm" software (GraphPad) to obtain k_{off} values: $P=V_s*t+(V_o-V_s)*(1^{-e(-koff*t)})/k_{off}$, where V_s = uninhibited enzyme velocity, measured in the reaction with enzyme and no inhibitor and V_o = inhibited enzyme velocity, measured in the reaction with no enzyme and NCF only.

Results

Figure 1. Expression level of KPC-2 wt and D179Y mutant in PAM1154 cells



- D179Y mutation had no effect on KPC-2 expression level in PAM1154 cells

Table 1. MIC values (µg/ml) for PAM1154 containing plasmids with the wild type KPC-2 or KPC-3 genes or the corresponding mutant proteins

Strain	Plasmid	Aztreonam	Imipenem	Meropenem	Cefepime	Ceftazidime
PAM4175	**pUCP24**	0.125	1	0.125	0.125	0.125
PAM4135	**pUCP24-KPC-2**	**128**	**16**	**64**	**256**	**32**
PAM4639	**pUCP24-KPC-2 D179Y**	**2**	1	**1**	**64**	**512**
PAM4689	**pUCP24-KPC-3**	**128**	**16**	**64**	**>256**	**128**
PAM4691	**pUCP24-KPC-3 D179Y**	**4**	0.5	**2**	**128**	**512**

Numbers in bold are for MICs that are at least 8-fold higher than that for the vector only strain

- KPC-3 differs from KPC-2 by a single H274Y substitution
- Consistent with recent literature reports, the KPC-3 containing strain was four-fold more resistant to ceftazidime as compared to KPC-2 transformant.
- D179Y mutation in both KPC-2 and KPC-3 resulted in significant changes in MIC values: a 16-64-fold **reduction** of aztreonam, imipenem and meropenem MICs and ≥ 4-fold reduction of cefepime MICs.
- D179Y introduced into KPC-2 or KPC-3 increased ceftazidime MICs 16-fold and 4-fold, respectively.

Table 2. MIC values (µg/ml) of ceftazidime alone or in combination with BLIs for PAM1154 containing plasmids with the wild type KPC-2 or KPC-3 genes or the corresponding mutant proteins

			Ceftazdime MIC with indicated BLI concentration (µg/ml)								
Strain	Plasmid	BLI	*0*	*0.5*	*1*	*2*	*4*	*8*	*16*	*32*	MPC_{64}*
PAM4135	**pUCP24-KPC-2**	**Vaborbactam**	**32**	2	1	0.5	0.5	0.5	0.5	0.5	**2**
PAM4639	**pUCP24-KPC-2 D179Y**	**Vaborbactam**	**512**	64	16	8	2	2	1	0.5	**2**
PAM4135	**pUCP24-KPC-2**	**Avibactam**	**32**	1	1	0.5	0.5	0.5	0.5	0.5	**2**
PAM4639	**pUCP24-KPC-2 D179Y**	**Avibactam**	**512**	128	128	64	32	16	4	2	**16**
PAM4689	**pUCP24-KPC-3**	**Vaborbactam**	**128**	16	2	1	0.5	0.5	0.5	0.5	**1**
PAM4691	**pUCP24-KPC-3 D179Y**	**Vaborbactam**	**512**	256	64	16	8	4	2	1	**4**
PAM4689	**pUCP24-KPC-3**	**Avibactam**	**128**	4	2	2	1	0.5	0.5	0.5	**1**
PAM4691	**pUCP24-KPC-3 D179Y**	**Avibactam**	**512**	256	128	64	32	16	8	4	**16**

* MPC_{64} is a concentration of a BLI to reduce MIC of an antibiotic 64-fold

- Vaborbactam and avibactam had similar potency in reducing ceftazidime MIC against KPC-2 and KPC-3 expressing strains as judged from similar MPC_{64} (1-2 µg/ml) and similar ceftazidime MIC with BLIs at 4 µg/ml, 0.5-1 µg/ml.
- Vaborbactam was more potent than avibactam in reducing ceftazidime MIC against D179Y mutants as judged from 4-8-fold lower MPC_{64} and 4-16-fold lower ceftazidime MIC with BLIs at 4 µg/ml

Table 3. Kinetic parameters of nitrocefin hydrolysis by KPC-2 and KPC-2-D179Y proteins

Enzyme	K_m, µM	k_{cat}, sec^{-1}	k_{cat}/K_m, sec^{-1}*µM^{-1}
KPC-2	35.7 ± 4.5	132 ± 13	3.7 ± 0.2
KPC-2 D179Y	34.6 ± 5.9	**0.25 ± 0.03**	0.0070 ± 0.0003

- K_m value of D179Y protein was very similar to that for the wild type KPC-2; NCF k_{cat} value exhibited almost 500-fold reduction.

Table 4. Ki values (µM) of vaborbactam and avibactam for the inhibition of nitrocefin hydrolysis mediated by KPC-2 and KPC-2-D179Y proteins

Enzyme	Vaborbactam	Avibactam
KPC-2 wt	0.059 ± 0.004	0.014 ± 0.002
KPC-2 D179Y	0.058 ± 0.013	0.011 ± 0.001

- K_i values of vaborbactam and avibactam for inhibition of nitrocefin hydrolysis were not affected by D179Y mutation, suggesting that this residue does not directly affect interaction with either of the BLIs.

Table 5. Kinetic parameters for the inhibition of nitrocefin hydrolysis mediated by the wild type KPC-2 and D179Y mutant protein by vaborbactam and avibactam

Enzyme	BLI	k_2/K (M^{-1}* s^{-1})	K_{off}, s^{-1}	Residence time, min	K_d, nM
KPC-2 wt	**Vaborbactam**	7.3 x 10^3	0.000052	318	7.2
KPC-2 D179Y	**Vaborbactam**	6.1 x 10^3	0.000056	296	9.2
KPC-2 wt	**Avibactam**	3.1 x 10^4	0.00026	64	8.3
KPC-2 D179Y	**Avibactam**	2.5 x 10^4	0.00021	78	8.5

- Similar to K_i values, all other kinetic parameters of vaborbactam and avibactam interaction with KPC-2 enzymes (k_2/K, k_{off}, residence time, K_d) were not affected by the D179Y mutation

Table 6. Kinetic parameters of ceftazidime hydrolysis by KPC-2 and mutant proteins

Enzyme	K_m, µM	k_{cat}, s^{-1}	k_{cat}/K_m, sec^{-1} * µM^{-1}, regular method	k_{cat}/K_m, sec^{-1} * µM^{-1}, new method	K_m, µM, calculated
KPC-2 wt	>2000	ND	ND	0.00087 ± 0.00011	ND
KPC-2 D179Y	<10	0.0088 ± 0.0003	ND	0.0088 ± 0.00093	1

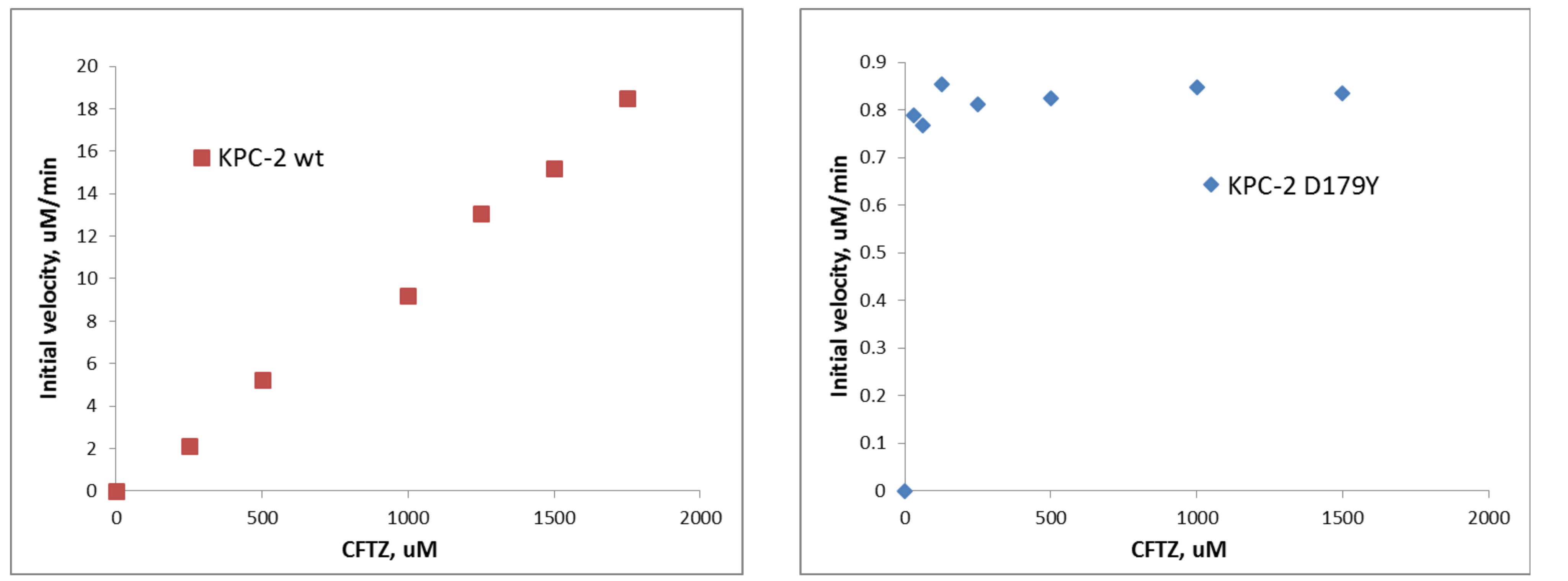
- k_{cat}/K_m catalytic ratio for the D179Y mutant is ten-fold higher than that for the wt KPC-2. This difference may explain higher ceftazidime MIC values conferred by this mutant.
- Calculated K_m value for ceftazidime for the D179Y mutant, 1 µM, (obtained by division of k_{cat} by k_{cat}/K_m) is significantly lower than that for the wt KPC-2.

Table 7. Kinetic parameters of inhibition of ceftazidime hydrolysis by KPC-2 and D179Y mutant protein

Enzyme	K_i (µM), Avibactam (10 min pre-incubation)	K_i (µM), Avibactam (no pre-incubation)	K_i, (µM), vaborbactam (10 min pre-incubation)	K_i (µM), Vaborbactam (no pre-incubation)
KPC-2 wt	0.20 ± 0.012	0.47 ± 0.04	0.33 ± 0.02	0.58 ± 0.19
KPC-2 D179Y	0.032 ± 0.0014	14.7 ± 0.9	0.015 ± 0.002	1.6 ± 0.3

- When avibactam and vaborbactam were pre-incubated with KPC-2 before addition of ceftazidime, their K_i values were similar, albeit slightly higher for vaborbactam. Both inhibitors also had similar K_i values for the D179Y mutant, but now K_i values for vaborbactam were slightly lower compared to that for avibactam. For both inhibitors, K_i values for D179Y mutant were lower, compared to that for the wild type KPC-2.
- When avibactam and vaborbactam were added to the wt KPC-2 at the same time as ceftazidime, their K_i values were also similar, and again, slightly higher for vaborbactam. However, in the presence of ceftazidime, avibactam K_i value for the D179Y mutant was 10-fold higher compared to that of vaborbactam. For both inhibitors, K_i values for D179Y mutant were higher, compared to that for the wild type KPC-2: avibactam apparent K_i for the D179Y mutant was increased 30-fold compared to wt KPC-2, while vaborbactam K_i was increased only 2.7-fold.
- The presence of ceftazidime did not significantly affect avibactam or vaborbactam K_is for the wt KPC-2 (1.7-2-fold effect), but it affected K_i s of both BLIs for the D179Y mutant. Avibactam K_i was affected ~4-fold stronger than that for vaborbactam.

Figure 2. Rates of ceftazidime hydrolysis plotted vs substrate concentration for wt KPC-2 and D179Y mutant



- Wild type KPC-2 demonstrated no sign of saturation of reaction velocity vs substrate concentration.
- D179Y mutant appears to have a low K_m value.

Conclusions

- Both microbiological and biochemical data demonstrate that the variants of KPC-2 and KPC-3 that carry D179Y mutation have increased catalytic efficiency for ceftazidime hydrolysis and cause resistance to avibactam but not vaborbactam inhibition
- Retention of vaborbactam inhibitory activity of KPC enzymes with the D179Y mutation associated with resistance to avibactam is consistent with a different mode of binding to the active site for vaborbactam compared to avibactam.

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Disclosures

Ruslan Tsivkovski and Olga Lomovskaya are employees of The Medicines Company that is developing vaborbactam in combination with meropenem for resistant gram-negative infections

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