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Cefpodoxime-ETX1317 Susceptibility is Unaffected by Ceftazidime-Avibactam Resistance Mutations V240G, D179Y and D179Y/T243M in KPC-3 β-Lactamase.

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Abstract

Introduction

ETX0282 is an orally bioavailable prodrug of the diazabicyclooctenone β-lactamase (BLA) inhibitor (BLI) ETX1317 in clinical development combined with cefpodoxime (CPD) proxetil for complicated urinary tract infections (cUTI). Avibactam (AVI) is a diazabicyclooctanone BLI already in clinical use combined with ceftazidime (CAZ-AVI). Mutations in KPC-2 and -3 BLAs that cause CAZ-AVI resistance have been identified[1-4]. We investigated the effect of CAZ-AVI resistance mutations in KPC-3 (V240G, D179Y, and D179Y/T243M) on enzymatic activity of KPC-3; on inhibition of KPC-3 by ETX1317 and avibactam; on susceptibility of isogenic BLA-expressing *Escherichia coli* strains to CPD-ETX1317 and CAZ-AVI; and on the covalent complex of KPC-3 with ETX1317.

Methods

MICs were measured according to CLSI guidelines, with CPD:ETX1317 in a fixed 1:2 ratio. Wild-type and mutant KPC-3 enzymes were cloned, overexpressed in *E. coli* and purified. Kinetic constants K_m and k_{cat} were measured for hydrolysis of nitrocefin, ceftazidime, and cefpodoxime. The kinetic constant k_{inact}/K_i was measured for inhibition by ETX1317 and avibactam. Isogenic strains of *E. coli* were prepared, each of which constitutively expressed wild-type or one of the KPC-3 variants as its only BLA. The amount of KPC-3 in each strain was quantified by Western blotting, showing that expression levels of all variants were all within 4-fold of each other. The masses of BLI adducts with KPC-3 variants were determined by intact protein mass spectrometry.

Results

MICs for CPD-ETX1317 in the isogenic strains were 0.12-0.25 mg/L for both wild-type and mutant KPC-3s, whereas all of the mutations decreased susceptibility to CAZ-AVI (MICs 2-16 mg/L). None of the CAZ-AVI resistance mutations in KPC-3 significantly affected k_{inact}/K_i for inhibition by ETX1317 or avibactam, but did affect kinetic constants for β-lactam hydrolysis. K_ms for nitrocefin and cefpodoxime were little affected, but K_m for ceftazidime was reduced 3-fold by D179Y and 85,000-fold by D179Y/T243M. k_{cat}s for all 3 substrates were little affected by V240G, but k_{cat} for CPD was reduced 16-fold in D179Y and 700-fold in D179Y/T243M. All KPC-3 variants formed 273 Da covalent adducts with ETX1317, corresponding to its full mass.

Conclusions

CAZ-AVI resistance mutations D179Y and D179Y/T243M in KPC-3 did not confer resistance to CPD-ETX1317 because k_{cat} for CPD decreased, K_m for CPD was unchanged, and k_{inact}/K_i by ETX1317 was unchanged compared with wild-type KPC-3. The kinetic constants for the V240G mutant were essentially the same as those of the wild-type enzyme.

Cefpodoxime — ETX1317 — Ceftazidime — Avibactam

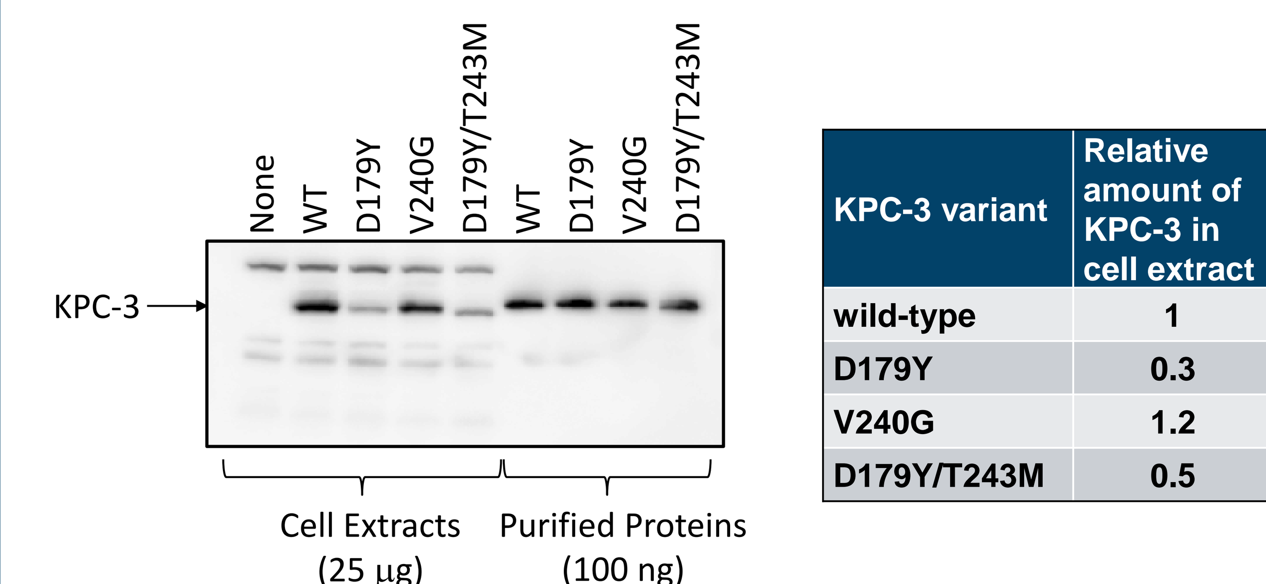
Minimal inhibitory concentrations (mg/L) of BLA and BLA/BLI combinations in isogenic *E. coli* strain panel expressing one KPC-3 variant

KPC-3 variant	CPD	ETX1317	CPD-ETX1317 (1:2)	Ceftazidime	Ceftazidime + 4 mg/L avibactam
None	2	16	0.125	0.5	0.25
wild-type	32	16	0.125	16	0.5
V240G	>64	16	0.25	64	2
D179Y	2	16	0.25	16	8
D179Y/T243M	2	16	0.125	32	16

- All 3 KPC-3 mutations significantly raised the CAZ-AVI MICs (4-32-fold) but not the CPD-ETX1317 MICs, compared to the MIC with wild-type KPC-3.
- The D179Y and D179Y/T243M mutants retained the ability to raise the CAZ MICs, but lost the ability to raise the CPD MICs.

Mutant KPC-3 expression in isogenic strains

Western blot of cell extracts with KPC-3-affinity-purified anti-KPC-2 antiserum



KPC-3 variant	Relative amount of KPC-3 in cell extract
wild-type	1
D179Y	0.3
V240G	1.2
D179Y/T243M	0.5

Band intensities from cell extracts were normalized for protein load based on the main non-specific band, and for the relative sensitivities of detecting purified KPC-3 variants.

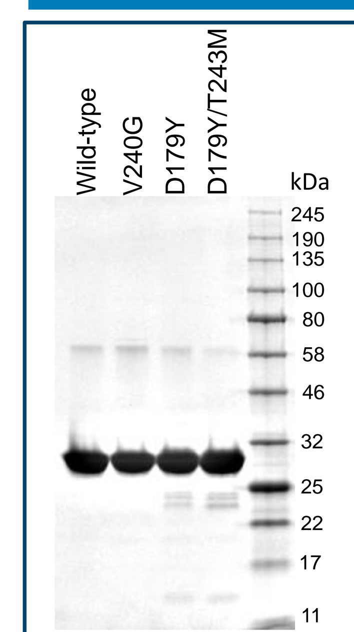
- Expression levels of D179Y and D179Y/T243M variants were 2-4-fold lower than wild-type and V240G variants.
- The differences between the expression levels of the KPC-3 variants do not account for the MIC effects observed in the isogenic panel.

Steady-state kinetic constants for hydrolysis of nitrocefin, ceftazidime, and cefpodoxime by KPC-3 variants, and second-order rate constants for inactivation of KPC-3 variants by ETX1317 and avibactam

	WT	V240G	D179Y	D179Y/T243M
Nitrocefin average ± std. dev. (n=3)				
K_m (µM)	45 ± 6	32 ± 4	34 ± 2	40 ± 10
k_{cat} (s^{-1})	71 ± 7	59 ± 5	1.5 ± 0.2	0.038 ± 0.002
k_{cat}/K_m ($M^{-1}s^{-1}$)	1.6 (± 0.1) x 10^6	1.8 (± 0.3) x 10^6	4.3 (± 0.5) x 10^4	1.0 (± 0.3) x 10^2
Ceftazidime average ± std. dev. (n=3)				
K_m (µM)	17,000 ± 3,000	17,000 ± 2,000	5,000 ± 1,000	0.2 ± 0.1
k_{cat} (s^{-1})	55 ± 7	300 ± 100	1.9 ± 0.6	0.006 ± 0.001
k_{cat}/K_m ($M^{-1}s^{-1}$)	3,300 ± 700	16,000 ± 5,000	380 ± 60	30,000 ± 10,000
Cefpodoxime average ± std. dev. (n=3)				
K_m (µM)	3,000 ± 1,000	4,000 ± 2,000	2,600 ± 200	1,800 ± 400
k_{cat} (s^{-1})	100 ± 30	170 ± 60	6.4 ± 0.4	0.14 ± 0.03
k_{cat}/K_m ($M^{-1}s^{-1}$)	35,000 ± 3,000	46,000 ± 3,000	2,430 ± 10	80 ± 2
k_{inact}/K_i ($M^{-1}s^{-1}$) average ± std. dev. (n=3)				
ETX1317	42,000 ± 4,000	32,000 ± 5,000	38,000 ± 6,000	28,000 ± 2,000
Avibactam	7,100 ± 700	4,700 ± 300	7,400 ± 300	5,900 ± 200

- None of the mutations substantially affected k_{inact}/K_i for AVI or ETX1317.
- ETX1317 was a more potent inhibitor than AVI of the KPC-3 variants.
- The V240G mutation moderately increased k_{cat} and k_{cat}/K_m for CAZ, but not CPD or nitrocefin.
- The D179Y mutation substantially decreased k_{cat} and k_{cat}/K_m for CAZ, CPD and nitrocefin, but only for CAZ was this somewhat offset by a reduced K_m.
- The D179Y/T243M double mutation greatly decreased k_{cat} for CAZ, CPD and nitrocefin as well as k_{cat}/K_m for nitrocefin and CPD. For CAZ, however, the decrease in k_{cat} was offset by a decrease in K_m such that k_{cat}/K_m was greatly increased.

Purified KPC-3

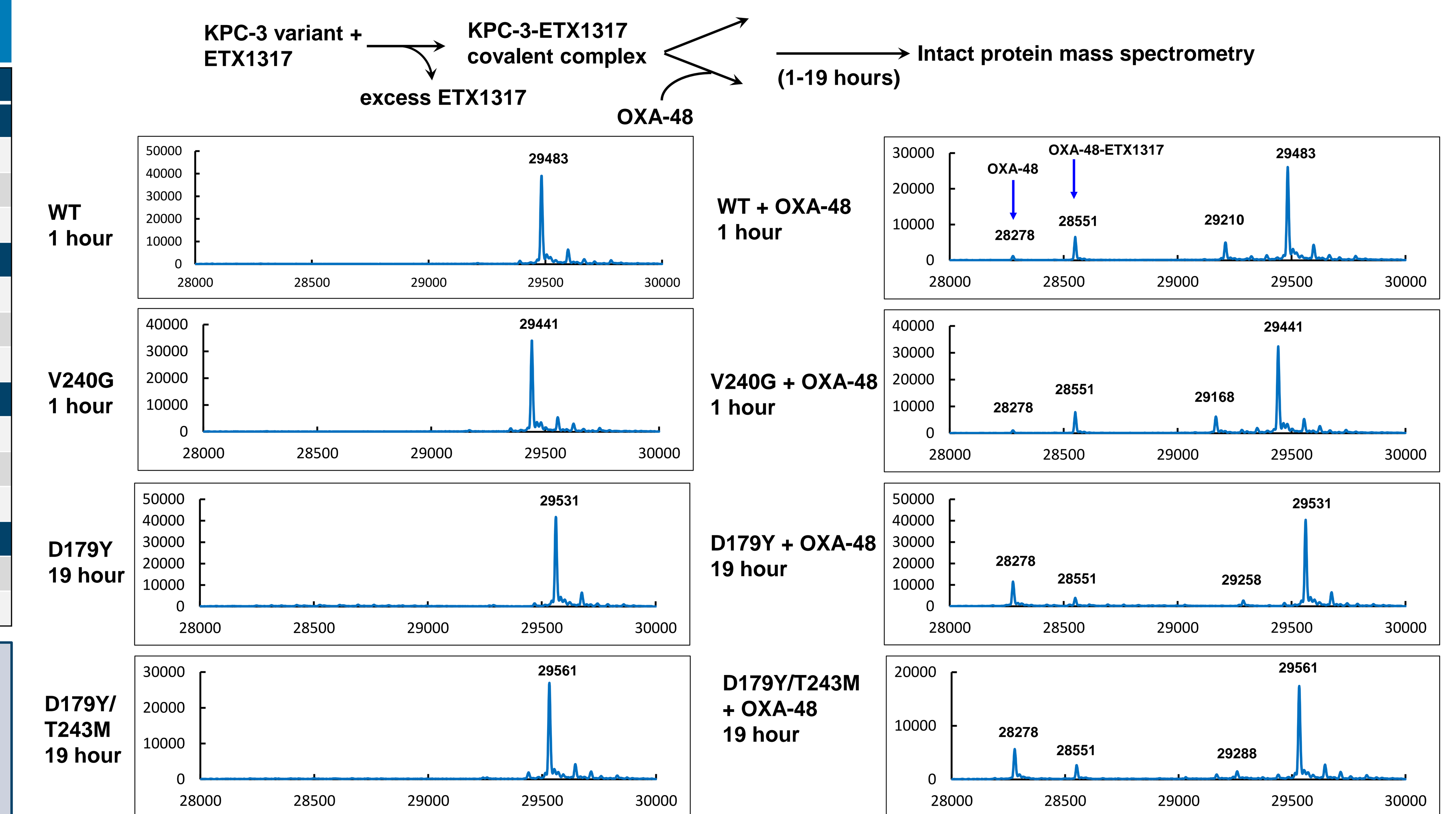


Masses of KPC-3 variants treated with ETX1317

KPC-3 variant	Inhibitor	Protein mass (Da)
wild-type	None	29,210
	ETX1317	29,483
V240G	None	29,168
	ETX1317	29,441
D179Y	None	29,258
	ETX1317	29,531
D179Y/T243M	None	29,288
	ETX1317	29,561

- All 4 KPC-3 variants reacted covalently with ETX1317, resulting in adducts with the full 273 Da mass of the inhibitor.

Re-cyclization and release of intact ETX1317 from KPC-3 variants detected by acylation exchange from KPC-3 to OXA-48 β-lactamase



- All of the KPC-3 variants formed covalent adducts with the full mass of ETX1317.
- None of the KPC-3 variants hydrolyzed covalently bound ETX1317 after 19 hours at room temperature.
- All 4 KPC-3 variants were able to exchange covalently bound ETX1317 with OXA-48, which means that ETX1317 recyclized and dissociated from KPC-3 in its original form.
- The rate of exchange from the D179Y and D179Y/T243M variants was much slower than from the wild-type and V240G variants, consistent with the lower k_{cat}s of the D179Y and D179Y/T243M variants.

References

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