VNRX-5133 (Taniborbactam), a broad-spectrum inhibitor of serine- and metallo-β-lactamases, restores activity of cefepime in Enterobacterales and Pseudomonas aeruginosa.

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Running title: Next-generation β-lactamase inhibitor taniborbactam.

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Abstract

As shifts in epidemiology of β-lactamase-mediated resistance continue, carbapenem-resistant *Enterobacterales* (CRE) and *Pseudomonas aeruginosa* (CRPA) are the most urgent threats. Although approved β-lactam-β-lactamase inhibitor (BL-BLI) combinations address widespread serine β-lactamases (SBLs) such as CTX-M-15, none provide broad coverage of both clinically important serine- (KPC, OXA-48) and metallo-β-lactamases (MBLs, e.g. NDM-1). VNRX-5133 (Taniborbactam) is a new cyclic boronate BLI in clinical development combined with cefepime for treatment of infections caused by β-lactamase-producing CRE and CRPA. Taniborbactam is the first BLI with direct inhibitory activity against Ambler class A, B, C and D enzymes. From biochemical and structural analyses, taniborbactam exploits substrate mimicry while employing distinct mechanisms to inhibit both SBLs and MBLs. It is a reversible covalent inhibitor of SBLs with slow dissociation and prolonged active site residence time \((t_{1/2} \text{ of } 30\text{-}105 \text{ min})\), while in MBLs, it behaves as a competitive inhibitor with \(K_i\) ranging from 0.019 to 0.081 µM. Inhibition is achieved by mimicking the transition state structure and exploiting interactions with highly conserved active site residues. In microbiological testing, taniborbactam restored cefepime activity in 33/34 engineered *E. coli* strains overproducing individual enzymes covering classes A, B, C and D, providing up to 1,024-fold shift in MIC. Addition of taniborbactam restored cefepime antibacterial activity in all 102 *Enterobacterales* and 38/41 *P. aeruginosa* clinical isolates with MIC\(_{90}\) of 1 and 4 µg/mL representing ≥256- and ≥32-fold improvement in antibacterial activity over cefepime alone. The data demonstrate potent, broad-spectrum rescue of cefepime activity in clinical isolates of CRE and CRPA.
There is an urgent need for new therapies to address the rise of infections caused by multidrug-resistant (MDR) gram-negative bacteria. Of particular concern are healthcare-associated infections by *Enterobacterales* and *P. aeruginosa*, where acquired resistance to reserved carbapenems significantly narrows therapeutic options (1-4). According to the 2019 CDC report, there are 210,500 cases per year of infections caused by extended-spectrum β-lactamase (ESBL)-producing *Enterobacterales* or carbapenem-resistant *Enterobacterales* (CRE) and 6,700 cases per year from multidrug-resistant *P. aeruginosa* (MDR-PA) infections in the United States resulting in 12,900 deaths annually (5). A 2017 study estimated the resistance burden within US inpatients as 290,000 ESBL, 170,000 MDR and 30,000 carbapenem non-susceptible *Enterobacterales* infections per year (6). No new antibiotic classes, particularly towards the challenging gram-negative pathogens, have been introduced since the fluoroquinolones. At the same time, serious resistance and safety concerns for several commonly used classes, including the fluoroquinolones (7-9) and polymyxins (10-11), have severely narrowed safe and effective options to treat these life-threatening infections (4).

β-lactams (e.g. penicillins, cephalosporins, monobactams and carbapenems) are the standard of care for most gram-negative infections (12). However, resistance conferred by β-lactamases continues to increase. More than 2,800 unique β-lactamases that span the spectrum of Ambler classes (A, B, C and D) have been identified, therein threatening the efficacy of β-lactams (13-14). Key among these are
the carbapenem-inactivating serine β-lactamases (SBLs) including KPC and OXA-48, and the emerging metallo-β-lactamases (e.g., NDM and VIM). CRE and CRPA producing SBLs and MBLs pose a serious challenge for infectious disease physicians and are a major public health concern (4, 15-18). One effective strategy to address the upsurge of carbapenemases is combination of a β-lactam (BL) with a β-lactamase inhibitor (BLI) to provide protection from these hydrolyzing enzymes (1, 2, 12). Although recently approved BL-BLI combinations (e.g. ceftazidime-avibactam (19-20), ceftolozane-tazobactam and meropenem-vaborbactam) (20-21) do offer protection from many SBLs, there are no approved BL-BLI combinations that are active against emerging metallo-β-lactamases (22-24). Within the SBLs, recent ceftazidime-avibactam and ceftolozane-tazobactam treatment failures of infections caused by Klebsiella pneumoniae or P. aeruginosa resulting from the production of KPC-3 or Pseudomonas-derived cephalosporinase (PDC) variants highlight the need for new agents that provide a broader spectrum of coverage (25-28). Early cyclic boronate inhibitors had been demonstrated to have significant potential to inhibit all classes of β-lactamases to enable improved broad-spectrum coverage (29-30).

A Venatorx Pharmaceuticals patent published in 2014 first disclosed the cyclic boronate BLI taniborbactam (formerly VNRX-5133) (Fig. 1) (31), whereas the discovery and medicinal chemistry optimization of taniborbactam were recently described (32). We present herein comprehensive biochemical, structural and microbiological data describing the broad-spectrum activity of taniborbactam in combination with the 4th generation cephalosporin, cefepime in comparison to recently-approved cephalosporin-BLI combinations. Our findings provide both the biochemical and structural basis for
broad-spectrum inhibition of β-lactamases by taniborbactam and show that addition of this next-generation BLI restores antibacterial activity of cefepime against Enterobacterales and P. aeruginosa producing clinically important SBLs and MBLs, including CTX-M-, KPC-, OXA-, NDM- and VIM-type β-lactamases.

Results

Biochemical and structural studies of taniborbactam, defining the mechanism of inhibition of both SBLs and MBLs. Kinetic parameters of taniborbactam (Fig. 1) relative to avibactam and vaborbactam including on-rates, off-rates and half-life of active site occupancy (t_{1/2}) with CTX-M-15 (Class A), KPC-2 (Class A) and P99 AmpC (Class C) are presented in Table 1. Inhibition behavior of all three BLIs with SBLs fits a two-step inhibition model in which a non-covalent complex forms followed by formation of a reversible covalent bond with the active site serine residue (Equation 1).

\[ \text{E} + \text{I} \xrightleftharpoons[k_{-1}]^{k_1} \text{EI} \xrightarrow[k_2]{k} \text{EI}^* \]  
(Equation 1)

Second order rate constants (k_2/K_i) of covalent bond formation to the active site serine of the three β-lactamases examined (CTX-M-15, P99 AmpC and KPC-2) were on the order of 10^4-10^5 M^{-1}s^{-1} for taniborbactam, relative to 10^3-10^5 for avibactam and 10^3 for vaborbactam (Table 1). Due to slow inhibitor off rates (k_{off} values ranging from 1.1 x 10^{-4} to 3.8 x 10^{-4} s^{-1}), taniborbactam exhibited a significant residence time within the active site with t_{1/2} values ranging from 30 to 105 minutes, relative to 29 to 249 minutes for avibactam, consistent with published data (33), and 5 to 32 minutes for vaborbactam (Table 1).
A 1.1-Å resolution X-ray co-crystal structure of taniborbactam and CTX-M-15 (PDB 6SP6) previously confirmed that the inhibitor binds covalently to the catalytic Ser70 with a bond distance of 1.53 Å, and that the boron atom adopts a tetrahedral conformation (Figure 2). Location of the boron hydroxyl group in the oxyanion hole suggests that the inhibitor acts as a mimic of the tetrahedral intermediate formed during the acylation step. Additionally, binding of taniborbactam displaces the deacylation water molecule (Wd) by 1.4 Å (Figure 2C) and exploits substrate-like interactions with conserved active site residues in serine-β-lactamases (Asn104, Ser130, Asn132, Asn170 and Thr235) (32).

Taniborbactam is distinguished from avibactam and vaborbactam by the ability to inhibit the most clinically relevant subclass B1 MBLs (VIM- and NDM-type enzymes). As observed in the co-crystal-structure of taniborbactam and VIM-2 (PDB 6SP7, Figure 3) (32), the boron atom adopts an sp³ hybridization state due to geminal diol formation after reacting with the active site hydroxide anion (the so-called bridging water coordinated to the Zn²⁺ active site cations). The boron hydroxyl interacts with Zn1 and both the conserved Asn233 and Asp120 residues. The carboxylate and oxygen atom of the cyclic oxaborinane interacts with Zn2, thus behaving as a mimic of the tetrahedral intermediate in subclass B1 enzymes. The substituted amino group of the inhibitor side chain interacts with Glu149, which is conserved in NDM-1. Interestingly, the inhibitor carboxylate does not interact with Arg228 but instead with the backbone of conserved Asn233. By comparing the bound structure with apoVIM-2 (PDB 1KO3) (34), binding of taniborbactam induces a narrowing of the active site cleft due to the approach of conserved Asn233 and Phe61 (Figure 3C). Finally, a surface rendering of VIM-2 bound...
by taniborbactam shows the presence of an electronegative pocket stabilizing the inhibitor side chain (Figure 3D), also providing a structural basis for inhibition of both VIM- and NDM-type MBLs, as residues constituting the pocket (Glu149 and Asp236) are conserved in both enzyme subgroups.

Inhibition of VIM-2 and NDM-1 by taniborbactam was reversible, as enzymatic activity was fully recovered after a rapid jump dilution. Using steady-state kinetic analysis, taniborbactam was confirmed as a competitive inhibitor of VIM-2 and NDM-1 with $K_i$ values of 0.019 and 0.081 µM respectively, whereas IMP-1 is outside of the spectrum of inhibition, with a $K_i$ greater than 30 µM (Table 2). Regarding the serine β-lactamases, taniborbactam had potent inhibitory activity against class A and C enzymes, with $K_i$ values ranging between 0.002 and 0.017 µM for SHV-5, KPC-2, CTX-M-15 and P99 AmpC, similar to avibactam. Against the class D OXA-48 enzyme, taniborbactam had a $K_i$ of 0.35 µM, similar to avibactam and vaborbactam (Table 2), and that level of potency was sufficient to protect cefepime from this β-lactamase subtype that exhibits weak cefepimase activity and consequently contributes minimally to cefepime resistance (Table 3) (35-36).

Potency and spectrum of activity defined in engineered E. coli overproducing individual β-lactamases. The spectrum of antibacterial activity of cefepime-taniborbactam and the breadth of inhibitory activity of this next generation cyclic boronate BLI were assessed in 34 engineered E. coli strains each overproducing an individual β-lactamase and directly compared to clinically-approved ceftazidime-avibactam. In this manner, antibacterial activity of the BL-BLI combinations relative to...
the partnered cephalosporins alone provided a quantitative measure of β-lactam potentiation that directly reflects the levels of inhibition of β-lactamase function achieved by addition of BLI. The BLI concentration was fixed at 4 µg/mL as described in Materials and Methods.

In strains overproducing Ambler class A β-lactamases, addition of taniborbactam potentiated cefepime activity from 8- to 1,024-fold, which is comparable to avibactam potentiation of ceftazidime (Table 3). Cefepime activity was potentiated by taniborbactam to within 4-fold of the vector control strain (FEP-TAN MIC of 0.12 µg/mL) in 16/17 class A β-lactamase overproducing strains, compared to only 4/17 for ceftazidime-avibactam (vector control strain CZA MIC of 0.25 µg/mL). Cefepime-taniborbactam provided potent coverage against clinically-derived serine β-lactamases associated with elevated MICs to ceftazidime-avibactam, including strains producing KPC-3 Ω loop variants (D179Y, V240G, A177E/D179Y and D179Y/T243M) (25-26), TEM-24, VEB-9 and PER-1/-2. This side-by-side comparison demonstrated differentiation through improved class A ESBL and KPC variant coverage by cefepime-taniborbactam.

In strains producing Ambler class B MBLs addition of taniborbactam potentiated the antibacterial activity of cefepime by 16- to 256-fold in all class B β-lactamase producing strains with the notable exception of IMP-1, where taniborbactam has insufficient inhibitory activity ($K_i \geq 30$ µM, Table 2) to potentiate the antibacterial activity of cefepime (Table 3). Addition of avibactam failed to restore ceftazidime activity in these strains, as would be expected from its lack of inhibitory activity against MBLs. By contrast, cefepime was highly potentiated (64- to 128-fold) by addition of taniborbactam.
against strains overproducing clinically-important NDM variants (NDM-1, -5 and -7).

This analysis therefore established the spectrum of MBL enzymes inhibited by taniboractam as SPM-1, GIM-1 and clinically-important variants of NDM (-1, -5 and -7) and VIM (-1, -2 and -4).

Among strains producing selected class C enzymes, cefepime-taniboractam MICs ranged from 0.12 to 0.5 µg/mL, reflecting an 8- to 32-fold potentiation of cefepime activity to within 4-fold of the vector control MIC. By contrast, ceftazidime-avibactam MICs were 0.5, 2 and 8 µg/mL in the ACT-17, ACT-C189 and CMY-2 overproducing strains, respectively. Although the former was within 2-fold of the vector control strain, the latter two were 8- and 32-fold higher than the vector control strain for ACT-C189 (P99 AmpC) and CMY-2, respectively, despite avibactam potentiating ceftazidime activity by 64- to 128-fold in these strains.

In strains overproducing class D β-lactamases (OXA-48, OXA-162, OXA-163, OXA-181, and OXA-232), cefepime activity was potentiated 8- to 512-fold by addition of taniboractam resulting in MICs of 0.12-0.25 µg/mL, within 2-fold of the vector control MIC. By comparison, addition of avibactam to ceftazidime had a 2-fold potentiation of ceftazidime activity in four of five strains resulting in MICs of 0.5 to 1 µg/mL in a similar range to those of cefepime-taniboractam. In the remaining class D β-lactamase producing strain, OXA-163 exhibited high ceftazidimase activity (CAZ MIC = 256 µg/mL) and although ceftazidime activity was potentiated 64-fold by addition of avibactam to an MIC of 4 µg/mL (16-fold higher than the vector control MIC), cefepime activity was potentiated 512-fold by addition of taniboractam providing a significantly improved MIC of 0.25 µg/mL, within 2-fold of the vector control MIC. From this side-by-side
comparison in class D β-lactamase overproducers, cefepime-taniborbactam and ceftazidime-avibactam appear to be equivalently active, though cefepime-taniborbactam exhibited better potency against OXA-163.

Overall, within this panel of 34 distinct β-lactamase overproducing strains, potentiation of cefepime MIC by taniborbactam ranged from 8- to 1024-fold in 33/34 strains, with a modal potentiation of 512-fold and MIC$_{50}$/MIC$_{90}$ values of 0.25/4 µg/mL. Thirty-three of 34 (97%) strains had cefepime-taniborbactam MICs of ≤4 µg/mL. The exception was the IMP-1 overproducer (cefepime alone MIC of 64 µg/mL; no potentiation by taniborbactam). By contrast, potentiation of ceftazidime MICs by avibactam ranged from 2- to 1024-fold in 25/34 strains, with a modal potentiation of 64-fold and MIC$_{50}$/MIC$_{90}$ values of 8/1,024 µg/mL. Nine of 9 (100%) MBL producing strains and 8 of 25 (32%) SBL producing strains fell outside of the ceftazidime-avibactam inhibitory spectrum, with MICs ≥16 µg/mL, providing a measure of differentiation for cefepime-taniborbactam against this challenge set of SBL overproducing strains encompassing Ambler class A, C and D β-lactamases.

**Antibacterial activity of cefepime-taniborbactam in reference isolates.**

Antibacterial activity of cefepime, cefepime-taniborbactam and taniborbactam alone was assessed against seven publicly available reference type isolates from the CDC, NCTC and ATCC (Table 4). This panel encompassed 3 *E. coli*, 2 *K. pneumoniae* and 2 *P. aeruginosa* isolates and included four quality control (QC) isolates for broth microdilution (37). Of particular note, taniborbactam demonstrated no clinically relevant antibacterial activity (MIC ≥512 µg/mL) within this panel. Among the β-lactamase-
producing Enterobacterales isolates, *E. coli* NCTC 13353 producing CTX-M-15 and *K. pneumoniae* BAA-1705 producing KPC-2 are routine QC isolates for cefepime-taniborbactam (37). Cefepime antibacterial activity was selectively potentiated by addition of 4 µg/mL taniborbactam in isolates producing β-lactamases with the exception of *P. aeruginosa* ATCC 27853 (*Pseudomonas*-derived cephalosporinase-5, PDC-5) against which cefepime alone is active (Table 4).

**Potentiation of cefepime activity by taniborbactam in clinical isolates of Enterobacterales and *P. aeruginosa***. The antibacterial activity of cefepime-taniborbactam was compared to ceftazidime-avibactam, ceftolozane-tazobactam and cefepime-tazobactam in a diverse panel of *Enterobacterales* and *P. aeruginosa* clinical isolates with defined β-lactamase subtypes (Table S1). IMP-producing strains were excluded as none of the BLI-protected cephalosporins inhibit IMP sufficiently to provide clinically-relevant rescue of the partner β-lactam, a characteristic also shared by approved BLI-protected carbapenems (meropenem-vaborbactam and imipenem-relabactam). The panel does not reflect current epidemiological trends but instead highlights differences in coverage provided by cefepime-taniborbactam relative to comparators. Antibacterial activity of the cephalosporins alone was included to ascertain the level of potentiation by the partnered BLI. BLIs were tested at a fixed concentration of 4 µg/mL in all combinations except cefepime-tazobactam, where tazobactam was fixed at a concentration of 8 µg/mL (38). The collection comprised 143 clinical isolates from 2005 and 2018. Isolates were subdivided by phenotypic profile with some level of molecular characterization into Enterobacterales producing: 1) mixed...
class A, class C and ESBLs, 2) serine- and metallo-carbapenemases (OXA-48/48-like, KPC, and NDM/VIM-type MBLs), or *P. aeruginosa* isolates producing: 3) basal levels of PDCs, downregulated OprD combined with upregulation of RND drug efflux systems, 4) ceftolozane-tazobactam-resistant PDC variants, and 5) serine- and metallo-carbapenemases (GES/KPC/VIM) (Table S1).

In 42 isolates of *Enterobacterales* (subdivision 1) expressing either mixed class A/C β-lactamases or ESBLs, cefepime-taniborbactam, cefepime-tazobactam and ceftazidime-avibactam had similar levels of activity with MIC<sub>50</sub>/MIC<sub>90</sub> values of 0.06/0.5, 0.12/1 and 0.5/1 µg/mL respectively, whereas MIC<sub>50</sub>/MIC<sub>90</sub> of ceftolozane-tazobactam was 4/32 µg/mL in this set of isolates (Figure 4A, Table S1).

In 60 *Enterobacterales* isolates (subdivision 2) expressing carbapenemases (OXA-48/48-like, KPC, or NDM-/VIM-metallo-β-lactamases), cefepime-taniborbactam was highly active with MIC<sub>50</sub>/MIC<sub>90</sub> of 0.5/2 µg/mL, relative to comparators ceftazidime-avibactam (2≥64 µg/mL), cefepime-tazobactam (32≥64 µg/mL), cefepime alone (64≥64 µg/mL) and ceftolozane-tazobactam (≥64≥64 µg/mL) (Figure 4B, Table S1).

Overall activity of the tested agents in 102 *Enterobacterales* isolates is summarized in Figure 4C (Table S1). MIC distributions of cefepime alone along with the four cephalosporin-BLI combinations are presented. Addition of taniborbactam reduced the MIC<sub>90</sub> from ≥256 µg/mL for cefepime alone to 1 µg/mL (Table S1). Cefepime-taniborbactam was the most active combination followed by ceftazidime-avibactam with MIC<sub>50</sub>/MIC<sub>90</sub> of 0.12/1 and 1≥64 µg/mL respectively (Figure 4C, Table S1).
In 14 *Pseudomonas aeruginosa* isolates (subdivision 3), ceftolozane-tazobactam (MIC\(_{90}\) = 4 \(\mu\)g/mL), cefepime-taniborbactam (MIC\(_{90}\) = 8 \(\mu\)g/mL) and ceftazidime-avibactam (MIC\(_{90}\) = 8 \(\mu\)g/mL) were all highly active. Cefepime-tazobactam was less active against these isolates with an MIC\(_{90}\) of 32 \(\mu\)g/mL (Figure 5A). The addition of taniborbactam reduced the cefepime MIC\(_{90}\) by ≥8-fold within this subset of isolates, similar to avibactam potentiation of ceftazidime activity. By contrast, addition of tazobactam reduced the MIC\(_{90}\) of ceftolozane by 4-fold and cefepime by 2-fold. Of note, one isolate in this subset, Paeβ-18, had upregulation of both MexAB-OprM (3.9-fold) and MexXY (5.6-fold) combined with 600-fold upregulation of PDC-3 expression providing MICs of 16, 32 and ≥64 \(\mu\)g/mL for ceftolozane-tazobactam, cefepime-taniborbactam and ceftazidime-avibactam respectively (39). The data suggest that the combined effect of drug efflux upregulation and highly elevated PDC variant production in *P. aeruginosa* can effectively reduce susceptibility to these cephalosporin-BLI combinations (Table S1).

Among 10 isolates of *P. aeruginosa* (subdivision 4) resistant to ceftolozane-tazobactam (MIC >8 \(\mu\)g/mL) through production of eight different PDC variants (Table S1), a single isolate remained susceptible to ceftazidime-avibactam with an MIC of 8 \(\mu\)g/mL, while all others had MIC values ranging from 16 to ≥ 64 \(\mu\)g/mL. Cefepime was more stable to these PDC variants than ceftazidime and the combination of cefepime-taniborbactam provided good antibacterial activity with MICs ranging from 2 to 16 \(\mu\)g/mL (Table S1).

In 17 *P. aeruginosa* (subdivision 5) producing KPC (n=3), GES (n=9) or VIM (n=5) carbapenemases, cefepime-taniborbactam was more active than comparator
cephalosporin/BLI combinations. MIC values for cefepime-taniboractam in all 17 isolates ranged between 1 and 8 µg/mL with an MIC\textsubscript{50} of 4 µg/mL and an MIC\textsubscript{90} of 8 µg/mL (Table S1). Ceftolozane-tazobactam activity was weak with MIC values ranging from 16 to ≥ 64 µg/mL. Ceftazidime-avibactam was active with MIC ≤ 8 µg/mL in 5/17 isolates including all three KPC-producers and two GES-6 producers, whereas all other isolates exhibited MICs ranging from 16 to ≥ 64 µg/mL. The data highlight a gap in coverage of the ceftazidime-avibactam combination in GES-producing \textit{P. aeruginosa} with 6/9 isolates having MICs ≥16 µg/mL (Table S1).

Overall in the 41 \textit{P. aeruginosa} isolates tested (subdivisions 3-5), addition of taniboractam to cefepime dramatically shifted the distribution of MICs to a lower range with an MIC\textsubscript{90} of 8 µg/mL compared to ≥256 µg/mL for cefepime alone (Figure 5B, Table S1). Ceftazidime-avibactam and ceftolozane-tazobactam had elevated MIC\textsubscript{90} of ≥ 64 µg/mL due to the production of VIM and GES variants along with cross-resistance to ceftazidime-avibactam within the ceftolozane-tazobactam-resistant isolates, consistent with previously published findings (27-28).

**Addition of taniboractam restores bactericidal activity of cefepime in NDM-1 producing \textit{K. pneumoniae} and VIM-2 producing \textit{P. aeruginosa} clinical isolates.** Restoration of bactericidal activity to cefepime by addition of taniboractam was further confirmed by time kill assays with \textit{K. pneumoniae} CDC-0049 producing NDM-1 and \textit{P. aeruginosa} Ps-12 producing VIM-2 (Figure 6). Both isolates were resistant to cefepime with MICs of 256 µg/mL and 32 µg/mL, respectively. Addition of taniboractam at a fixed concentration of 4 µg/mL shifted the cefepime MIC to 4 µg/mL in both cases. In
both isolates, cefepime-taniborbactam achieved a 3-Log\textsubscript{10} reduction in CFU/mL relative to starting inoculum by 6 h without regrowth thereafter. By contrast, ceftazidime-avibactam tested at 32 µg/mL (4-fold above CLSI susceptibility breakpoint of 8 µg/mL) had little to no impact on either isolate due to high MICs originating from a lack of coverage of VIM-/NDM-type MBLs.

**Resistance to cefepime-taniborbactam.** Frequency of resistance (FoR) studies were performed at 4×MIC of cefepime with taniborbactam fixed at 4 µg/mL against 8 Enterobacterales and *P. aeruginosa* strains expressing Ambler Class A, B, C and D β-lactamases. The FoR ranged from 1.6 x 10\textsuperscript{-9} to 8.9 x 10\textsuperscript{-11} (Table 5), indicating a low potential for spontaneous development of resistance to cefepime-taniborbactam among target pathogens. Moreover, no single step taniborbactam-resistant β-lactamase variants were obtained from this standard first pass investigation.

**High selectivity for β-lactamases.** The selectivity and specificity of taniborbactam were evaluated in the DrugMatrixScreen\textsuperscript{TM} panel of pharmacological targets at Eurofins/Panlabs. Binding, enzymatic and uptake assays representing a wide range of cellular and subcellular target classes were performed. At the screening concentration of 100 µM, no notable off-target findings (≤23% inhibition) were reported in 128 of 129 in vitro assays. The one exception where significant inhibition (>50%) was observed was with a β-lactamase.

**Discussion**
The goal of a β-lactamase inhibitor in a BL-BLI combination is to rescue the β-lactam from degradation by the complement of β-lactamases present, thereby restoring its activity to that seen in the absence of β-lactamase enzymes, and essentially restoring MICs to a wild-type distribution. The BL-BLI approach was effectively introduced with the first generation BLIs tazobactam, sulbactam and clavulanic acid paired with the penicillins, piperacillin, ampicillin and amoxicillin, respectively. This strategy has been extended more recently to protected cephalosporins (ceftazidime-avibactam and ceftolozane-tazobactam) and carbapenems (meropenem-vaborbactam and imipenem-relebactam). The focus of these recently approved BL-BLI combinations had been to address the growing concerns regarding KPC-type enzymes. With the advent of protected cephalosporins and carbapenems, there is an opportunity to re-establish stratification of β-lactams into front-line protected cephalosporins (ceftazidime-avibactam, ceftolozane-tazobactam) and to reserve carbapenems (e.g., meropenem-vaborbactam, imipenem-relebactam) (40). Over the past decade, other problematic β-lactamase expansions in Enterobacterales and P. aeruginosa have emerged particularly in select geographies, including most notably widespread OXA-48/OXA-48-like β-lactamase producing Enterobacterales in Europe (18, 41), VIM-2 MBL producing ST-235 P. aeruginosa in Belarus, Kazakhstan and Russia (42) and NDM-1/5/7 MBL producing Enterobacterales in India and China (43-45). None of the recently-approved BL-BLI combinations is sufficiently active against Enterobacterales or P. aeruginosa isolates producing MBLs, and meropenem-vaborbactam also lacks coverage for OXA-48 (46). Taniborbactam, which is being developed in combination with cefepime for the treatment of complicated urinary tract infections (cUTI) and hospital-acquired or...
ventilator-associated bacterial pneumonia (HABP/VABP), represents the next stage of this approach. We have now demonstrated uniquely potent activity of cefepime-tanibor- 
tobactam against both SBLs and MBLs, and especially against these emerging MBLs and OXA-48/OXA-48-like SBLs compared to ceftazidime-avibactam, ceftolozane-
tazobactam and cefepime-tazobactam.

In addition to emerging MBLs and OXA-48/OXA-48-like SBLs, reports of antibiotic resistance evolving during the course of clinical therapy with BL-BLIs including to ceftazidime-avibactam in KPC-producing *Enterobacterales* and with ceftolozanetazobactam in *P. aeruginosa* producing altered PDC variants are increasing. Most concerning with the PDC variants is cross-resistance to ceftazidime-avibactam is often co-associated (27-28). The activity of cefepime-tanibor- 

tobactam against these resistant variants of PDC and KPC would importantly provide a future alternative therapeutic option to address evolving resistance. Cefepime-tanibor- 
bactam thus has the potential, once approved for clinical use, to provide the broadest coverage of β-lactamases to complement the existing repertoire of anti-gram-negative agents in the clinic and help address these problematic β-lactamase expansions by providing additional optionality for physicians to effectively treat these infections.

**Materials and Methods**

**Expression plasmid construction.** Plasmid DNA, PCR product purification and gel extractions were performed using Wizard Plus SV miniprep and SV gel and PCR extraction kits (Promega). *NdeI*, *BamHI* and *XhoI* restriction enzymes, T4 DNA ligase and *E. coli* BL21(DE3) competent cells were purchased from New England Biolabs. All
oligonucleotide primers for PCR amplification were purchased from Integrated DNA technologies. All PCR reactions were performed with Phusion high fidelity DNA polymerase and cloning performed in E. coli DH5α sub-cloning efficiency chemically-competent cells (ThermoFisher). The pET9a (Agilent) and pET24a (MilliporeSigma) expression clones were made using PCR amplification products from molecularly characterized clinical isolates carrying the desired β-lactamase gene and cloned Ndel to BamHI into pET9a in all cases except blaNDM-1 that was cloned Ndel to XhoI into pET-24a. All β-lactamases were cloned with signal peptide encoding sequences except NDM-1 that lacked the coding sequence for the first 28 amino acids encompassing the signal peptide and the lipobox (LSGC) peptide sequence. All transformants were verified by PCR amplification, restriction endonuclease mapping and DNA sequencing. Confirmed expression plasmids were isolated by plasmid mini-prep and used to transform the expression cell lines E. coli BL21(DE3) or E. coli JM109(DE3).

Construction of isogenic strains of E. coli producing individual β-lactamases. The isogenic strains were used to establish spectrum of inhibitory activity against 34 β-lactamases that were engineered into E. coli DH5α cells, carrying chromosomal AmpC (ESC-1), a non-ESBL enzyme unable to hydrolyze these third or fourth generation cephalosporins. Each β-lactamase gene region encoding the periplasmic protein was placed under the control of the blaTEM-1 promoter and signal peptide sequence to drive expression and localization of β-lactamases to the periplasm. The DNA fragments containing the promoter, the signal sequence, and each β-lactamase coding gene were synthesized and cloned into pTwist Chlor High Copy in.
DH5α at Twist Bioscience. The individual expression plasmids along with their Genbank accession numbers are listed in Table S2. The protein sequences of β-lactamases were obtained from the Beta-Lactamase DataBase (www.bldb.eu) (47). Expression of the β-lactamases was confirmed by verifying decreased susceptibility to example test antibiotics that are known substrates of the enzymes. *E. coli* DH5α carrying pTU501 that expressed only the TEM-1 signal sequence from the *bla* promoter was used as a control. The plasmid sequences are available through GenBank (Table S2).

**β-lactamase purification.** For all β-lactamases except P99 AmpC, a 50 mL pre-culture of *E. coli* BL21(DE3) cells containing the pET-based expression vector (pET-24a for NDM-1 and pET9a otherwise) for the individual β-lactamases was grown overnight in Lysogeny Broth (LB) medium at 37 °C in the presence of kanamycin selection at 50 µg/mL. Common to all β-lactamase purifications, bacterial cells were lysed by three consecutive passes through a chilled French Pressure Cell at 18,500 psi and clarified by centrifugation at 10,000 × g for 30 minutes at 4 °C. The β-lactamase activity was monitored using nitrocefin at 100 µM and purity was examined by 10% SDS-PAGE with Coomassie Brilliant Blue staining. Purified proteins exhibiting >95% purity by SDS-PAGE were quantified by Pierce BCA protein assay kit and bovine serum albumin as standard (ThermoFisher), concentrated to a working range of 1 to 5 mg/mL and frozen at -80 in buffer containing 10% glycerol. Column purifications were performed using an AKTA FPLC (GE Healthcare). The purification schemes were generally similar with enzyme-specific differences described below.

CTX-M-15 was obtained from *E. coli* BL21(DE3) carrying plasmid pET-CTX-M-15, grown in 2L of MagicMedia autoinduction medium (Invitrogen) containing 50 µg/mL
kanamycin (Sigma) for 24 h at 23 °C. Cells were harvested at A_{600nm} of 2.2 by centrifugation at 7,500 × g at 4 °C, re-suspended in 60 mL of 10 mM Hepes pH 7 supplemented with 0.5 mM EDTA. The lysate was diluted 5-fold with cold 50 mM sodium acetate pH 4.8 and incubated overnight at 4 °C. The extract was clarified by centrifugation at 14,500 × g at 4 °C, filtered through an Amicon nitrogen concentrator with a 10 kDa cutoff filter to a volume of 50 mL and loaded onto a Hitrap CaptoS column pre-equilibrated in 50 mM sodium acetate pH 4.8. Protein was eluted by a linear gradient of 50 mM sodium acetate pH 4.8 supplemented with 500 mM NaCl. Fractions containing active CTX-M15 were pooled, concentrated and the buffer exchanged for 20 mM Hepes pH 7.2, 150 mM NaCl and 10% glycerol using Amicon Ultra-15 centrifugal concentrators. CTX-M-15 was further separated by a Superdex 200 gel filtration column.

OXA-48 was obtained from E. coli BL21(DE3) carrying plasmid pET-OXA-48, grown in 2 L of MagicMedia autoinduction medium (Invitrogen) with kanamycin as previously described for 24 h at 23 °C. Cells were harvested at A_{600nm} of 3 by centrifugation at 7,500 × g at 4 °C, re-suspended in 60 mL of 20 mM triethanolamine pH 5.5, then purified as described for CTX-M-15 with the exception that the final buffer contained 10 mM NaHCO₃ to maintain the critical active site lysine residue carbamylated (48).

KPC-2 was obtained from E. coli BL21(DE3) carrying plasmid pET-KPC-2, grown in 3 L of MagicMedia autoinduction medium (Invitrogen) with kanamycin as previously described for 24 h at 23 °C. Cells were harvested at A_{600nm} of 3.3 by centrifugation at 7,500 × g at 4 °C, re-suspended in 70 mL of 20 mM MES pH 5.5. The extract was
clarified by centrifugation at 14,500 x g at 4 °C, filtered through an Amicon nitrogen concentrator by use of 10 kDa cutoff filters to a volume of 50 mL and loaded onto a Hitrap CaptoS column pre-equilibrated in 20 mM MES pH 5.5. Protein was eluted by a linear gradient of 20 mM MES pH 5.5 supplemented with 500 mM NaCl. KPC-2 active fractions were pooled, concentrated and buffer exchanged in 20 mM Hepes pH 7.3, 150 mM NaCl using Amicon Ultra-15 centrifugal concentrators. KPC-2 was further separated by a gel filtration chromatography with a Superdex 200 column.

P99 AmpC was purified directly from an Enterobacter cloacae SIP9925 P99+ clinical isolate after sequence of the β-lactamase encoding gene had been verified by DNA sequencing of PCR amplified product. To produce P99 AmpC, Enterobacter cloacae P99+ cells were grown in LB in the presence of a sub-MIC concentration (0.015 µg/mL) of imipenem to induce maximal expression of the enzyme. Cells were harvested at A_{600nm} of 2.4 by centrifugation at 7,500 x g at 4 °C, re-suspended in 50 mL of 20 mM MES pH 5.0 and otherwise purified in a similar manner to KPC-2 described above.

SHV-5 was obtained from E. coli BL21(DE3) pLysS carrying plasmid pET-SHV-5, grown in 2 L of Super broth at 25 °C to an A_{600nm} of 0.5, when IPTG was added to 0.05 mM final and induction proceeded for 6 h. Cells were harvested by centrifugation at 5,500 g for 15 min at 4 °C. The cell pellet was re-suspended in 60 ml of 10 mM HEPES buffer pH 7.5 and cells were lysed by French Press. The lysate was diluted 5-fold in 50 mM sodium acetate buffer pH 5 and kept at 4 °C overnight. The extract was clarified by centrifugation at 14,500 x g at 4 °C, filtered through an Amicon nitrogen concentrator by use of 10 kDa cutoff filters to a volume of 50 mL and loaded onto a HiTrap CaptoS
equilibrated with 50 mM sodium acetate buffer pH 5. SHV-5 was eluted by a linear gradient of 50 mM sodium acetate pH 5 supplemented with 500 mM NaCl. Active fractions were pooled, concentrated and buffer exchanged in 20 mM HEPES pH 7.3, 150 mM NaCl and 10% glycerol using Amicon Ultra-15 centrifugal concentrators. Finally, the SHV-5 sample was further purified by chromatography over a Superdex 200 gel filtration column.

VIM-2 was purified from *E. coli* BL21(DE3) carrying pET-VIM-2 as previously described (49) with the following changes, MagicMedia autoinduction medium was used instead of BSB broth with IPTG induction, cells were harvested at $A_{600\text{nm}}$ of 2.1 by centrifugation at 7,500 × g, for 15 min at 4 °C, the 50-80% ammonium sulphate precipitate was collected by centrifugation at 13,000 × g for 1 h at 4 °C and solubilized 20 mM HEPES pH 7.2, 50 µM ZnSO$_4$ at 1/20 of the original volume, loaded onto a 30 mL Q sepharose anion exchange column (GE Healthcare) equilibrated with the same buffer. Elution of VIM-2 was achieved with a linear NaCl gradient to 1 M. Fractions containing VIM-2 were pooled and concentrated with Amicon Ultra15 centrifugal concentrators with 10 kDa cutoff. The VIM-2 was dialyzed in 50 mM Tris pH 9, 50 µM ZnSO$_4$ overnight, loaded onto a MonoQ column pre-equilibrated in the same buffer and elution was achieved by linear gradient of NaCl up to 500 mM. Active fractions containing VIM-2 were pooled, concentrated and buffer exchanged into 50 mM HEPES pH 7.5, 50 µM ZnSO$_4$, 200 mM NaCl and further separated by gel filtration with a Superdex 200 column (GE Healthcare) equilibrated in the same buffer.

IMP-1 was purified from *E. coli* BL21(DE3) carrying plasmid pET-IMP-1 grown in 3L LB at 37°C with 50 µg/mL kanamycin selection as previously described with minor
modifications (50). At an A_{600nm} of 0.7, IPTG was added to a final concentration of 0.5 mM, and induction of IMP-1 expression was allowed to proceed for an additional 5 h at 30 °C. Cells were centrifuged at 7,500 × g for 15 minutes at 4 °C. The pellet was resuspended in 50 mM HEPES pH 7 containing 50 µM ZnSO₄. The lysate was clarified by centrifugation at 12,500 × g for 30 minutes at 4 °C and loaded onto a HiTrap Capto S column equilibrated in 50 mM HEPES pH 7 50 µM ZnSO₄. The column was washed with the same buffer, and the enzyme was eluted by a linear gradient of NaCl gradient to 500 mM. The active fractions were pooled and concentrated by ultrafiltration with Amicon centrifugal concentrators (10kDa cutoff). The protein solution was dialyzed in 50 mM HEPES pH 7 50 µM ZnSO₄ and loaded onto a MonoS cation exchange column that had been pre-equilibrated the same buffer. The enzyme was eluted with a linear gradient of NaCl to 500 mM. Fractions containing active IMP-1 were collected, pooled, and concentrated to 1 mg/ml.

NDM-1 lacking the signal peptide (first 28 amino acids) and lipobox sequence was purified as a soluble protein from the cytoplasm of Escherichia coli JM109 (DE3) carrying plasmid pET-NDM-SP, grown in 3 L of MagicMedia with kanamycin selection as described above. Protein expression was induced by addition of 0.5 mM IPTG at an A_{600nm} of 0.5 followed by incubation at 23 °C for 18 h. Cells were harvested by centrifugation at 7,500 × g for 15 min at 4 °C and washed twice with 25 mM Tris-HCl pH 7, 50 µM ZnSO₄. Cell lysates obtained by French Press were clarified by centrifugation at 12,500 × g for 30 min at 4 °C. The supernatant was loaded onto a Q sepharose column equilibrated in 25 mM Tris-HCl pH7, 50 µM ZnSO₄ and enzyme was eluted by a linear gradient of the same buffer supplemented with 500 mM NaCl. Active fractions of
NDM-1 were pooled, concentrated and loaded onto a Superdex 200 gel filtration column equilibrated in 25 mM Tris-HCl pH7, 50 µM ZnSO₄, 150 mM NaCl. NDM-1 containing fractions of high purity were pooled and dialyzed into 10 mM HEPES pH7 containing 20 µM ZnSO₄ and 0.01 mg/mL bovine serum albumin (BSA).

**Reversible Inactivation of Serine Active Site β-lactamases.** The kinetic parameters associated with reversible inactivation of CTX-M-15, KPC-2 and P99 AmpC were assessed by monitoring CTX-M-15-mediated cephalothin hydrolysis, KPC-2-mediated imipenem hydrolysis and P99 AmpC-mediated cephalothin hydrolysis spectrophotometrically at 37 °C in 50 mM sodium phosphate buffer (pH 7.0) within the first 15 minutes of reaction. To measure hydrolysis rates we used the following extinction coefficients (Δε): cephalothin Δε, -6,300 M⁻¹ cm⁻¹ at 273 nm; imipenem Δε, -9,000 M⁻¹ cm⁻¹ at 299 nm. For KPC-2, 500 µL reactions were initiated by addition of 6.25 pmoles KPC-2 and were performed in quadruplicate with 75 µM imipenem and six concentrations of taniborbactam (2.5 µM; 3.3 µM; 4 µM; 4.8 µM; 5.5 µM and 6.3 µM).

For P99 AmpC, reactions were initiated by addition of 1.25 pmoles of enzyme and performed in quadruplicate at 37 °C with 50 µM cephalothin and six concentrations of taniborbactam (0.125, 0.18, 0.26, 0.37, 0.52 and 0.75 µM). For CTX-M-15, reactions were initiated by addition of 3 pmoles of enzyme and performed in quadruplicate at 37 °C with 70 µM cephalothin and six concentrations of taniborbactam (0.75, 0.915, 1.12, 1.36, 1.66 and 2.03 µM). Similar concentration ranges were tested for both avibactam and vaborbactam. A reversible two-step inhibition model was fit to the data.
Pseudo first order rate constants of enzyme inactivation ($k_{obs}$) were determined in the presence of various inhibitor concentrations by fitting Equation 2 to the time courses.

\[ P = V_s t + (V_0 - V_s) \left(1 - e^{-kt}\right) \]

(Equation 2)

A plot of $k_{obs}$ versus inhibitor concentrations generated a linear plot with a slope of \((k_2/K_i)\) and a Y-intercept of $k_2$ (Equation 3). Reported values have been corrected for substrate concentration and $K_M$ for each substrate-enzyme combination as defined by the \((1 + [S]/K_M)\) term in equation 4 used to obtain the second order rate constant $k_2/K_i$ and the off-rate $k_2$. Error values reported are standard errors from the fit.

\[ k_{obs} = k_2 + \frac{k_2}{K_i} \left(1 + \frac{[S]}{K_M}\right) \]

(Equation 3)

A plot of the fractional steady state velocities showed a linear relationship with inhibitor concentrations tested from which an equilibrium dissociation constant ($K_i^*$ - Equation 4) was derived (Equation 3).

\[ K_i^* = \frac{K_i k_2}{k_2 + k_2} \]

(Equation 4)

Imipenem and cefotaxime were used as substrates for determining $K_i^*$ of taniborbactam with OXA-48 and SHV-5, respectively. Taniborbactam was tested in triplicate from 0.050 to 5 µM with OXA-48 (2.5 nM) and from 0.4 to 100 nM with SHV-5 (5 nM) in 50 mM sodium phosphate (pH 7.0). Similar concentrations ranges were tested with avibactam and vaborbactam.
Off-rates ($k_2$) were assessed by a jump dilution method in triplicate reactions monitored continuously for recovery of nitrocefinase enzymatic activity ($\Delta \varepsilon$, -20,500 M$^{-1}$ cm$^{-1}$ at 486 nm). CTX-M-15 (2 µM), KPC-2 (4 µM) and P99 AmpC (1 µM) were inactivated by taniborbatam at 10, 20 and 5 µM respectively. Similar concentration ranges were tested with avibactam and vaborbactam. A 4000-fold jump dilution was performed for the β-lactamase-taniborbatam reaction mixtures. Enzyme activity was monitored continuously by absorbance at 486 nm after the final 10-fold dilution consisting of the addition of 20 µL of inactivation reaction to 180 µL of 1 x PBS pH 7.4, 0.1 mg/mL BSA at 37 °C and nitrocefin at 150 µM final concentration. The percentage enzyme activity at each assay point was determined by comparison to enzyme in the absence of inhibitor and recovery of enzymatic activity was fit to a single exponential with associated standard deviation.

**Inhibition of VIM-2 and NDM-1 MBLs.** The inhibition modality of taniborbatam with both VIM-2 and NDM-1 metallo-β-lactamases was assessed by monitoring the impact of varying concentrations of taniborbatam on Michaelis-Menten kinetic parameters $K_M$ and $V_{\text{max}}$. Time-dependence of inhibitory activity was examined by pre-incubation of enzyme with inhibitor up to 30 minutes prior to initiation of reactions and recovery of enzymatic activity was assessed by jump dilution method as previously described (33). For VIM-2, triplicate 150 µL reactions were initiated by addition of the substrate nitrocefin ranging in concentration from 11.5 to 200 µM and contained 141 ng (4.5 pmoles) of VIM-2 tested against five concentrations of taniborbatam (5, 10, 20, 40 and 80 nM) and monitored at 486 nm using a Biotek Powerwave XS2 UV/Vis
spectrophotometric plate reader. By contrast, for NDM-1, triplicate 300 µL reactions were initiated by addition of the substrate cefotaxime ranging in concentration from 13.1 to 150 µM and contained 622.4 ng (24 pmoles) of NDM-1 tested against seven concentrations of taniborbactam (6, 12, 24, 48, 72, 96 and 120 nM) and substrate hydrolysis was monitored at 260 nm. Avibactam and vaborbactam showed no significant inhibition up to 50 µM. Nitrocefin was used as substrate for assays with IMP-1. No pre-incubation of enzyme and inhibitor was required for either VIM-2 or NDM-1. Inhibition modality was ascertained by global fitting of the Michaelis-Menten equation for competitive inhibition (Equation 5) to all the data, from which $K_i$ values with VIM-2 and NDM-1 were calculated.

$$V_o = \frac{V_{max}[S]}{[S] + K_m(1 + \frac{[I]}{K_i})}$$

(Equation 5)

**Antibacterial Activity.** The *in vitro* antibacterial activity of β-lactams alone or in combination with BLIs was determined in cation-adjusted Mueller-Hinton (CAMHB) broth microdilution assays according to CLSI recommendations (51). Antibacterial potentiation by taniborbactam, avibactam or tazobactam was assayed by fixing the BLI at 4 µg/mL with cefepime, ceftazidime, and ceftolozane respectively, while tazobactam was added at a fixed concentration of 8 µg/mL to cefepime as described by Wockhardt Pharmaceuticals. Inocula for broth microdilution assays were prepared by the broth culture method (51). The β-lactams were 2-fold serially diluted in CAMHB broth with final concentrations generally ranging between 0.06 and 128 µg/mL when tested alone.
and from 0.016 to 32 µg/mL when tested in combination with their respective BLIs. MICs reported are modal values from 5 independent replicates performed on separate days in either engineered *E. coli* DH5α producing individual β-lactamases or in clinical isolates of *Enterobacterales* and *P. aeruginosa*.

**Justification for the use of a fixed concentration of 4 µg/mL of taniborbactam for in vitro testing.** *In vitro* activity of cefepime-taniborbactam is measured as activity of cefepime in the presence of taniborbactam at a fixed concentration of 4 µg/mL. The rationale for choosing 4 µg/mL of taniborbactam for *in vitro* testing was based on *in vitro* broth microdilution and time-kill assessments, correlation of results in non-clinical models of infection, and PK-PD modeling.

Taniborbactam alone lacks antibacterial activity. At a fixed concentration of 4 µg/mL taniborbactam, the MIC<sub>90</sub> of cefepime was reduced from >128 to 2 µg/mL for cefepime non-susceptible *Enterobacterales* isolates, with 97.6% inhibited at 8 µg/mL of cefepime (52). This value corresponds to the susceptible, dose dependent (SDD) breakpoint for cefepime (37). The MIC distribution of cefepime-taniborbactam with taniborbactam fixed at 4 µg/mL against cefepime-non-susceptible *Enterobacterales* from surveillance similarly resembles the MIC distribution of cefepime against cefepime-susceptible isolates from the same study (52). Overlapping MIC distributions for cefepime against cefepime-susceptible strains and cefepime-taniborbactam against cefepime-non-susceptible strains (with taniborbactam fixed at 4 µg/mL) reflects complete or near-complete rescue of cefepime by taniborbactam against target organisms. Importantly, humanized dosing of cefepime and taniborbactam in the
neutropenic murine thigh infection model reduced the bacterial burden of all isolates of *Enterobacterales* and *P. aeruginosa* with cefepime-taniborbactam MIC values of 8 µg/mL or below by at least 1 log₁₀ (53). These results are consistent with the MIC values for cefepime derived in the presence of 4 µg/mL of taniborbactam.

**Isolation of avibactam and ceftolozane from clinical presentation.** Avibactam was isolated from commercially available ceftazidime-avibactam (AVYCAZ). A single bottle of AVYCAZ (containing 2 g of ceftazidime and 0.5 g of avibactam) was dissolved in approximately 6-8 mL of water and purified using a Biotage 120 g, C18 reverse phase column. A gradient utilizing a 99:1 mixture of H₂O (with 0.1% TFA):acetonitrile (ACN) (with 0.1% TFA) for four column volumes (CV) followed by a ramp to 1:100 H₂O/ACN over 1 CV was employed. All fractions containing avibactam were combined, frozen, and lyophilized to provide pure avibactam. Electrospray ionization Mass spectrometry (ESI-MS) confirmed m/z of 266.1 (M+H)+.

Ceftolozane was isolated from commercially available ceftolozane-tazobactam (ZERBAXA). A single bottle of ZERBAXA (containing 1 g of ceftolozane and 0.5 g tazobactam) was dissolved in water (~6-8 mL) and purified using a Biotage SNAP Ultra C18, 120g reverse phase column. A gradient beginning with 5 CVs of 95:5 H₂O (with 0.1% TFA)/ACN (with 0.1% TFA) was followed by a ramp to 80:20 H₂O/ACN over 6 CVs. Collected fractions were combined, frozen, and lyophilized. The isolated solid was triturated with 4.0 N HCl in diethyl ether and concentrated three times to provide ceftolozane as the hydrochloride salt. ESI-MS confirmed m/z of 667.1 (M+H)+.
**In vitro antibacterial kill assay.** Bactericidal activity was assessed by time-kill assay according to standard CLSI methods (54). Cefepime concentrations to be assayed in combination with taniborbactam were selected based on antibacterial activity (MIC), while ceftazidime-avibactam was tested at 32 μg/mL, (4x the CLSI susceptibility breakpoint of 8 μg/mL) (37). Time-kill assays were performed in 14 mL glass tubes with a bacterial inoculum in CAMHB broth of 5 x 10⁵ CFU/mL. The tubes were incubated at 37 °C with shaking at 200 rpm and aliquots were drawn at six time points (0, 2, 4, 6, 8 and 24 hours) to conduct 0.5 Log₁₀ dilutions in CAMHB broth in 96 well plates with activated charcoal. From these dilution plates, 10 μL were spotted onto Omnitrays using a replicator. Both Omnitrays and dilution plates were incubated overnight at 37 °C and used to ascertain viable bacterial counts. The lowest quantifiable amount by this method was 2 Log₁₀ CFU.

**Spontaneous frequency of resistance.** Five colonies were picked from agar plates with an inoculating loop and transferred aseptically to a glass Erlenmeyer flask containing 20 mL of CAMHB. The culture was grown ≥ 5 hours at 37 °C with shaking at 200 rpm. CAMHB agar (15g/L Becton Dickinson Difco agar) was prepared according to the manufacturer’s directions. Cefepime was added at 4x the MIC along with taniborbactam at a fixed concentration of 4 μg/mL. The agar was dispensed into 100 mm petri dishes (~20 mL per dish), 100 plates per test organism. Agar plates without drug were used to obtain colony counts. Optical density of growing cultures for inoculation were assessed spectrophotometrically at 600 nm. Inoculums were adjusted to a final suspension of 1 x 10⁸ CFU/mL to 1 x 10⁹ CFU/mL. Inoculum (100 μL) was
spread onto each plate with an inoculating loop. Plates were incubated at 37 °C and
colony counts were assessed at 24 and 48 hours.

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Figure legends

Figure 1: Structure of taniboractam (VNRX-5133).

Figure 2: A - Overall fold of the superimposed native CTX-M-15 (cyan, PDB code 4HBT) (55) and its covalent complex with taniboractam (green, PDB code 6SP6) (32);
B - Active site close-up and omit map of the taniboractam-bound CTX-M-15 complex;
C - Mode of binding of taniborbactam in the active site of the class A ESBL CTX-M-15, showing the main interactions between the enzyme and taniborbactam (magenta); taniborbactam interacts with many conserved residues of serine-\(\beta\)-lactamases (Asn104, Ser130, Asn132, Asn170, Thr235); when compared to the structure of the native CTX-M15 (PDB code 4HBT), the deacylation water molecule (Wd) is displaced by 1.4 Å upon inhibitor binding. Wa refers to acylation water and SO\(_4\) is sulfate from crystallization buffer solution. Figures were prepared with CCP4mg (56) or PyMol (https://pymol.org).

**Figure 3:** A - Overall fold of the superimposed native VIM-2 metallo-\(\beta\)-lactamase (cyan, PDB code 1KO3) (34) and its covalent complex with taniborbactam (green, PDB code 6SP7) (32); B - Active site close-up and omit map of the taniborbactam-bound VIM-2 complex; C - Mode of binding of taniborbactam in the active site of the VIM-2 metallo-\(\beta\)-lactamase, showing the main interactions between the enzyme and VNRX-5133 (magenta). Residues were numbered according to the BBL consensus numbering scheme (57).; D - Surface rendering of the VIM-2 active site in the VNRX-5133-inhibited complex, showing an inhibitor-induced narrowing of the active site cleft, resulting from a closer contact between the side chains Phe61 and Asn233; the presence of the electronegative pocket interacting with the inhibitor side chain, and contributing to the stability of the inhibitor-enzyme complex, is also shown.

**Figure 4:** MIC distributions from broth microdilution testing of cefepime-taniborbactam and comparators in *Enterobacterales*. Number of isolates at each MIC for cefepime relative to cefepime-tazobactam, cefepime-taniborbactam, ceftazidime-avibactam and...
ceftolozane-tazobactam with BLI fixed at 4 µg/mL in all cases except for FEP-TZB, where BLI was fixed at 8 µg/mL. %S, defined as MIC ≤ 8 µg/mL for FEP alone, FEP-TZB, FEP-TAN and CZA, or C/T with MIC ≤ 2 µg/mL in Enterobacterales. FEP = cefepime, FEP-TZB = cefepime-tazobactam, FEP-TAN = cefepime-tanibobactam, CZA = ceftazidime-avibactam and C/T = ceftolozane-tazobactam. A- Enterobacterales producing mixed class A/C and extended-spectrum β-lactamases (N=42). MIC$_{50}$/MIC$_{90}$ values were 8/128, 0.12/1, 0.06/0.5, 0.5/1 and 4/32 µg/mL for FEP, FEP-TZB, FEP-TAN, CZA and C/T respectively. %S were 61.9%, 100%, 100%, 100% and 42.9% respectively. B- Enterobacterales producing carbapenemases including OXA-48/OXA-48-like, KPC- and metallo-β-lactamases (N=60). MIC$_{50}$/MIC$_{90}$ values were 64/≥256, 32/≥64, 0.5/2, 2/≥64 and ≥64/≥64 µg/mL for FEP, FEP-TZB, FEP-TAN, CZA and C/T respectively. %S were 5%, 38.3%, 100%, 65% and 3.3% respectively. C- Overall distribution of MICs in all 102 isolates of Enterobacterales class A and C, OXA-48/OXA-48-like, ESBLs, KPC and VIM- and NDM-type β-lactamases. MIC$_{50}$/MIC$_{90}$ values were 32/≥256, 2/≥64, 0.12/1, 1/≥64 and 32/≥64 µg/mL for FEP, FEP-TZB, FEP-TAN, CZA and C/T respectively. %S were 28.4%, 63.7%, 100%, 79.4% and 19.6% respectively.

Figure 5: MIC distributions from broth microdilution testing in P. aeruginosa. Number of isolates at each MIC for cefepime relative to cefepime-tazobactam, cefepime-tanibobactam, ceftazidime-avibactam and ceftolozane-tazobactam with BLI fixed at 4 µg/mL in all cases except for FEP-TZB, where BLI was fixed at 8 µg/mL. %S, defined as MIC ≤ 8 µg/mL for FEP alone, FEP-TZB, FEP-TAN and CZA, or C/T with MIC ≤ 4 µg/mL in P. aeruginosa. FEP = cefepime, FEP-TZB = cefepime-tazobactam, FEP-TAN =
cefepime-taniborbactam, CZA = ceftazidime-avibactam and C/T = ceftolozane-tazobactam. A- *P. aeruginosa* with wild-type basal PDC expression or downregulated OprD combined with upregulated RND drug efflux systems and PDC variant expression levels (N=14). MIC\textsubscript{50}/MIC\textsubscript{90} values were 8/≥64, 8/32, 4/8, 4/8 and 2/4 µg/mL for FEP, FEP-TZB, FEP-TAN, CZA and C/T respectively. %S were 50%, 71.4%, 92.9%, 92.9% and 92.9% respectively. B- Overall distribution of MICs in 41 isolates of *P. aeruginosa* producing wild-type PDCs with varying levels of OprD and MexAB-OprM/MexXY-OprM efflux pumps, PDC variants affecting activity of ceftolozane-tazobactam, and KPC, GES or VIM carbapenemases. MIC\textsubscript{50}/MIC\textsubscript{90} values were 32/≥256, 16/≥64, 4/8, 16/≥64 and 32/≥64 µg/mL for FEP, FEP-TZB, FEP-TAN, CZA and C/T respectively. %S were 22%, 29.3%, 92.7%, 46.3% and 31.7% respectively.

**Figure 6.** Time-kill curves for cefepime-taniborbactam relative to ceftazidime-avibactam in two metallo-β-lactamase producing clinical isolates. The log\textsubscript{10} of the viable CFU/mL is displayed on the Y axis versus time (hours) on the X axis. Curves for 1×, 2× and 4×MIC for cefepime with taniborbactam fixed at 4 µg/mL are shown for each strain while the curves for ceftazidime at 32 µg/mL with avibactam fixed at 4 µg/mL, representing 0.015×MIC (*K. pneumoniae* CDC-0049) and 0.5×MIC (*P. aeruginosa* PS-12) for ceftazidime, but well above the CLSI susceptibility breakpoint for ceftazidime-avibactam in *Enterobacterales* and *P. aeruginosa* of 8 µg/mL.
| β-lactamase inhibitor | Kinetic parameters | Properties of taniborbactam with β-lactamases |
|-----------------------|-------------------|---------------------------------------------|
|                       | $k_d/K_i$ (10^4 M⁻¹ s⁻¹) | CTX-M-15 | KPC-2 | P99 AmpC |
| Taniborbactam          | $k_d/K_i$ (10^4 M⁻¹ s⁻¹) | 2.1 ± 0.1 | 0.9 ± 0.1 | 17.2 ± 0.8 |
|                       | $k_{off}$ (s⁻¹)     | 3.4 ± 0.2 | 1.1 ± 0.1 | 3.8 ± 0.3 |
|                       | $t_{1/2}$ (min)     | 34 ± 2 | 105 ± 5 | 30 ± 3 |
| Avibactam              | $k_d/K_i$ (10^4 M⁻¹ s⁻¹) | 10.8 ± 0.6 | 1.2 ± 0.1 | 0.32 ± 0.01 |
|                       | $k_{off}$ (s⁻¹)     | 4 ± 0.1 | 1.8 ± 0.1 | 0.5 ± 0.04 |
|                       | $t_{1/2}$ (min)     | 29 ± 1 | 66 ± 4 | 249 ± 19 |
| Vaborbactam            | $k_d/K_i$ (10^4 M⁻¹ s⁻¹) | 0.11 ± 0.01 | 0.12 ± 0.01 | 0.18 ± 0.01 |
|                       | $k_{off}$ (s⁻¹)     | 23 ± 0.9 | 5.4 ± 0.5 | 3.7 ± 0.3 |
|                       | $t_{1/2}$ (min)     | 5 ± 0.2 | 21 ± 2 | 32 ± 3 |


### TABLE 2 $K_i$ values for taniborcactam with various β-lactamases.

| β-lactamase | Class | Inhibitor constant $K_i$ (µM)\(^*\) |
|-------------|-------|--------------------------------------|
|             |       | Taniborcactam | Avibactam | Vaborbactam |
| SHV-5       |       | 0.003 ± 0.0002 | n.d.      | n.d.        |
| CTX-M-15    | A     | 0.017 ± 0.002 | 0.011 ± 0.001 | 0.158 ± 0.006 |
| KPC-2       |       | 0.004 ± 0.001 | 0.0056 ± 0.0007 | 0.022 ± 0.002 |
| NDM-1       |       | 0.081 ± 0.003 | >30        | >30         |
| VIM-2       | B     | 0.019 ± 0.001 | >30        | >30         |
| IMP-1       |       | >30          | >30        | >30         |
| P99 AmpC    | C     | 0.002 ± 0.0003 | 0.013 ± 0.0003 | 0.053 ± 0.004 |
| OXA-48      | D     | 0.35 ± 0.007  | 0.26 ± 0.005  | 0.35 ± 0.007  |

\(^*\) $K_i$ for Ambler Class A, C and D and $K_i$ for Ambler class D as described in Materials and Methods. n.d. Not determined.
Table 3 Spectrum of antibacterial activity of cefepime-taniborbactam defined in engineered strains of E. coli producing individual Class A, B, C and D β-lactamases

| E. coli DH5α pTU-501 expressing | Ambler Class | MIC (µg/mL) | Fold potentiation | MIC (µg/mL) | Fold potentiation |
|---------------------------------|-------------|-------------|-------------------|-------------|-------------------|
| Vector control                  | N/A         | 0.5         | 0.25              | 2           | 0.12              |
| TEM-10                          |             | 1024        | 2                 | 512         | 8                 |
| TEM-24                          |             | 1024        | 16                | 64          | 2                 |
| TEM-72                          |             | 1024        | 1                 | 1024        | 32                |
| CTX-M-2                         |             | 32          | 0.5               | 64          | 128               |
| CTX-M-15                        |             | 128         | 1                 | 128         | 128               |
| GES-5                           |             | 64          | 4                 | 16          | 4                 |
| SHV-5                           |             | 1024        | 4                 | 256         | 128               |
| VEB-9                           |             | 1024        | 16                | 64          | 128               |
| KPC-2                           |             | 64          | 1                 | 64          | 64                |

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| E. coli DH5α | Ambler Class | MIC (µg/mL) | Fold potentiation | MIC (µg/mL) | Fold potentiation |
|-------------|-------------|-------------|-------------------|-------------|-------------------|
| pTU-501 expressing CAZ | CAZ | CAZ activity | FEP | FEP-TAN | FEP activity |
| KPC-3 | 512 | 4 | 128 | 128 | 0.25 | 512 |
| KPC-3(D179Y) | 1024 | 128 | 8 | 32 | 1 | 32 |
| KPC-3(V240G) | 1024 | 32 | 32 | 256 | 0.5 | 512 |
| KPC-3(T243A) | 256 | 8 | 32 | 64 | 0.12 | 512 |
| KPC-3(A177E/D179Y) | 1024 | 512 | 2 | 16 | 0.5 | 32 |
| KPC-3(D179Y/T243M) | 1024 | 256 | 4 | 16 | 0.5 | 32 |
| PER-1 | 1024 | 32 | 32 | 512 | 0.5 | 1024 |
| PER-2 | 1024 | 128 | 8 | 256 | 0.5 | 512 |
| NDM-1 | 1024 | 1024 | 1 | 256 | 4 | 64 |
| NDM-5 | B | >1024 | >1024 | 1 | 512 | 4 | 128 |
| NDM-7 | >1024 | >1024 | 1 | 512 | 4 | 128 |
| VIM-1 | 1024 | 1024 | 1 | 128 | 2 | 64 |
| Parameter of antibacterial activity | E. coli DH5α pTU-501 expressing | Fold | MIC (µg/mL) | Fold | MIC (µg/mL) |
|------------------------------------|----------------------------------|------|-------------|------|-------------|
|                                    | Ambler                           |      | CAZ         |      | FEP         |
|                                    | Class                            |      | CZA         |      | FEP-TAN     |
|                                    | CAZ activity                     |      | FEP activity|      |             |
| VIM-2                              | 128                              | 1    | 128         | 1    | 16          |
| VIM-4                              | 256                              | 1    | 256         | 1    | 32          |
| IMP-1                              | B                                | 1    | 1024        | 1    | 64          |
| SPM-1                              | 1024                             | 1    | 1024        | 1    | 64          |
| GIM-1                              | >128                             | 1    | >128        | 1    | 4           |
| CMY-2                              | 512                              | 64   | 8           | 16   | 2           |
| ACT-C189 (P99 AmpC)                | C                                |      | 2           |      | 8           |
| ACT-17                             | 8                                | 16   | 0.5         |      | 4           |
| OXA-48                             | 1                                | 2    | 0.5         | 2    | 2           |
| OXA-162                            | D                                | 2    | 1           | 2    | 8           |
| OXA-163                            | 256                              | 64   | 4           |      | 128         |
| OXA-181                            | 1                                | 2    | 0.5         | 2    | 2           |

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| E. coli DH5α Class | MIC (µg/mL) | Fold potentiation | MIC (µg/mL) | Fold potentiation |
|-------------------|-------------|-------------------|-------------|-------------------|
| pTU-501 expressing | CAZ         | 1                 | 0.5         | 2                 |
|                   | CZA         |                   | CAZ activity|                   |
|                   | FEP         |                   | FEP         |                   |
|                   | FEP-TAN     |                   | TAN         |                   |
|                   | FEP activity|                   |             |                   |

Abbreviations: µg/mL = microgram, FEP = cefepime, mL = milliliter, TAN = taniborbactam, CAZ = ceftazidime, CZA = ceftazidime-avibactam. Taniborbactam and avibactam were tested in combination with cefepime and ceftazidime respectively at a fixed concentration of 4 µg/mL. Modal MIC values from five independent replicates are reported.
TABLE 4. Antibacterial activity of cefepime or taniborbactam alone relative to cefepime-taniborbactam combination in quality control reference type isolates.

| Strain         | ID        | Enzyme Content | MIC (µg/mL) |
|----------------|-----------|----------------|-------------|
|                |           |                | FEP | FEP-TAN | TAN |
| E. coli        | ATCC 25922| None           | 0.06 | 0.06    | 512 |
| E. coli        | NCTC 13353| CTX-M-15       | 64   | 0.25    | 512 |
| E. coli        | CDC-0452  | NDM            | 64   | 0.25    | 512 |
| K. pneumoniae  | ATCC 13883| None           | 0.06 | 0.06    | 512 |
| K. pneumoniae  | BAA 1705  | KPC-2          | 16   | 0.25    | 1024|
| P. aeruginosa  | ATCC 27853| PDC-5          | 1    | 1       | 1024|
| P. aeruginosa  | CDC-0457  | VIM            | 16   | 4       | 1024|

Abbreviations: µg/mL = microgram, FEP = cefepime, mL = milliliter, TAN = taniborbactam. Taniborbactam was tested in combination with cefepime at a fixed concentration of 4 µg/mL. Modal MIC values are...
reported. MICs for FEP-TAN in routine QC isolates *E. coli* ATCC 25922, *E. coli* NCTC 13353, *K. pneumoniae* BAA 1705 and *P. aeruginosa* ATCC 27853 are within acceptable QC ranges (58).

### Table 5. Spontaneous frequency of resistance to cefepime-taniborbactam in select gram-negative bacteria.

| Strain          | ID       | Enzyme content | MIC \(^1\) (µg/mL) | Total CFU | Frequency of Resistance \(^2\) |
|-----------------|----------|----------------|--------------------|-----------|-------------------------------|
| *E. coli*       | 25922    | AmpC           | 0.03               | 2.19 x 10\(^{10}\) | 9.1 x 10\(^{-11}\)           |
| *P. aeruginosa* | 27853    | PDC-5          | 2                  | 2.5 x 10\(^{9}\)   | <4 x 10\(^{-10}\)           |
| *K. pneumoniae* | BAA 1705 | KPC-2          | 0.125              | 4.6 x 10\(^{9}\)   | 6.5 x 10\(^{-10}\)           |
| *E. cloacae*    | ECL01    | P99 AmpC       | 0.06               | 1.31 x 10\(^{10}\) | 8.9 x 10\(^{-11}\)           |
| *E. coli*       | ESBL 4   | CTX-M15, TEM-1 | 0.06               | 4.1 x 10\(^{9}\)   | 2.4 x 10\(^{-10}\)           |
| *K. pneumoniae* | SI-117   | VIM-1          | 0.25               | 2.4 x 10\(^{9}\)   | <4.2 x 10\(^{-10}\)          |
| *E. coli*       | SI-152   | NDM-1          | 0.125              | 9 x 10\(^{9}\)     | 1.6 x 10\(^{-9}\)           |
| *E. coli*       | VER      | OXA-48         | 0.03               | 4.8 x 10\(^{9}\)   | 4.2 x 10\(^{-10}\)           |

\(^1\)MIC of cefepime titrated with taniborbactam fixed at 4 µg/mL. \(^2\)Agar plates contained Cefepime at 4x MIC and taniborbactam fixed at 4 µg/mL. Calculated as: (CFU observed in the presence of cefepime-taniborbatcam)/(total CFU).
Figure 1. Structure of taniborbactam (VNRX-5133).
Figure 2: A - Overall fold of the superimposed native CTX-M-15 (cyan, PDB code 4HBT) (55) and its covalent complex with taniborbactam (green, PDB code 6SP6) (32); B - Active site close-up and omit map of the taniborbactam-bound CTX-M-15 complex; C - Mode of binding of taniborbactam in the active site of the class A ESBL CTX-M-15, showing the main interactions between the enzyme and taniborbactam (magenta); taniborbactam interacts with many conserved residues of serine-β-lactamases (Asn104,
Ser130, Asn132, Asn170, Thr235); when compared to the structure of the native CTX-M-15 (PDB code 4HBT), the deacylation water molecule (Wd) is displaced by 1.4 Å upon inhibitor binding. Wa refers to acylation water and SO$_4$ is sulfate from crystallization buffer solution. Figures were prepared with CCP4mg (56) or PyMol (https://pymol.org).
**Figure 3:** A - Overall fold of the superimposed native VIM-2 metallo-β-lactamase (cyan, PDB code 1KO3) (34) and its covalent complex with taniborbactam (green, PDB code 6SP7) (32); B - Active site close-up and omit map of the taniborbactam-bound VIM-2 complex; C - Mode of binding of taniborbactam in the active site of the VIM-2 metallo-β-lactamase, showing the main interactions between the enzyme and taniborbactam (magenta). Residues were numbered according to the BBL consensus numbering scheme (57). D - Surface rendering of the VIM-2 active site in the taniborbactam-inhibited complex, showing an inhibitor-induced narrowing of the active site cleft, resulting from a closer contact between the side chains Phe61 and Asn233; the presence of the electronegative pocket interacting with the inhibitor side chain, and contributing to the stability of the inhibitor-enzyme complex, is also shown.
Figure 4A: MIC distributions in *Enterobacterales* producing mixed class A/C and extended-spectrum β-lactamases (N=42). Number of isolates at each MIC for cefepime relative to cefepime-tazobactam, cefepime-taniborbactam, ceftazidime-avibactam and ceftolozane-tazobactam with BLI fixed at 4 µg/mL in all cases except for FEP-TZB, where BLI was fixed at 8 µg/mL. MIC$_{50}$/MIC$_{90}$ values were 8/128, 0.12/1, 0.06/0.5, 0.5/1 and 4/32 µg/mL for FEP, FEP-TZB, FEP-TAN, CZA and C/T respectively. %S, defined as MIC ≤8 µg/mL for FEP alone, FEP-TZB, FEP-TAN and CZA, or C/T with MIC ≤2 µg/mL were 61.9%, 100%, 100%, 100% and 42.9% respectively. FEP = cefepime, FEP-TZB = cefepime-tazobactam, FEP-TAN = cefepime-taniborbactam, CZA = ceftazidime-avibactam and C/T = ceftolozane-tazobactam.
**Figure 4B:** MIC distributions in *Enterobacterales* producing carbapenemases including OXA-48/OXA-48-like, KPC- and metallo-β-lactamases (N=60). Number of isolates at each MIC for cefepime relative to cefepime-tazobactam, cefepime-taniborcactam, ceftazidime-avibactam and ceftolozane-tazobactam with BLI fixed at 4 µg/mL in all cases except for FEP-TZB, where BLI was fixed at 8 µg/mL. MIC$_{50}$/MIC$_{90}$ values were 64/≥256, 32/≥64, 0.5/2, ≥64/≥64 µg/mL for FEP, FEP-TZB, FEP-TAN, CZA and C/T respectively. %S, defined as MIC ≤8 µg/mL for FEP alone, FEP-TZB, FEP-TAN and CZA, or C/T with MIC ≤2 µg/mL were 5%, 38.3%, 100%, 65% and 3.3% respectively. FEP = cefepime, FEP-TZB = cefepime-tazobactam, FEP-TAN = cefepime-taniborcactam, CZA = ceftazidime-avibactam and C/T = ceftolozane-tazobactam.
**Figure 4C**: Overall distribution of MICs in all 102 isolates of *Enterobacterales* class A and C, OXA-48/OXA-48-like, ESBLs, KPC and VIM- and NDM-type β-lactamases.

Number of isolates at each MIC for cefepime relative to cefepime-tazobactam, cefepime-taniborbactam, ceftazidime-avibactam and ceftolozane-tazobactam with BLI fixed at 4 µg/mL in all cases except for FEP-TZB, where BLI was fixed at 8 µg/mL.

MIC\(_{50}/\)MIC\(_{90}\) values were 32/≥256, 2/≥64, 0.12/1, 1/≥64 and 32/≥64 for FEP, FEP-TZB, FEP-TAN, CZA and C/T respectively. %S, defined as MIC ≤8 µg/mL for FEP alone, FEP-TZB, FEP-TAN and CZA, or C/T with M\(IC ≤ 2 \mu g/mL\) were 28.4%, 63.7%, 100%, 79.4% and 19.6% respectively. FEP = cefepime, FEP-TZB = cefepime-tazobactam, FEP-TAN = cefepime-taniborbactam, CZA = ceftazidime-avibactam and C/T = ceftolozane-tazobactam.
Figure 5A: MIC distributions from broth microdilution testing in *P. aeruginosa* characterized by wild-type basal PDC expression or downregulated OprD combined with upregulated RND drug efflux systems and PDC variant expression levels (N=14).

Number of isolates at each MIC for cefepime relative to cefepime-tazobactam, cefepime-taniborbactam, ceftazidime-avibactam and ceftolozane-tazobactam with BLI fixed at 4 µg/mL in all cases except for FEP-TZB, where BLI was fixed at 8 µg/mL.

MIC₅₀/MIC₉₀ values were 8/≥64, 8/32, 4/8, 4/8 and 2/4 µg/mL for FEP, FEP-TZB, FEP-TAN, CZA and C/T respectively. %S, defined as MIC ≤ 8 µg/mL for FEP alone, FEP-TZB, FEP-TAN and CZA, or C/T with MIC ≤4 µg/mL were 50%, 71.4%, 92.9%, 92.9% and 92.9% respectively. FEP = cefepime, FEP-TZB = cefepime-tazobactam, FEP-TAN = cefepime-taniborbactam, CZA = ceftazidime-avibactam and C/T = ceftolozane-tazobactam.
Figure 5B: Overall MIC distributions in 41 isolates of P. aeruginosa producing wild-type PDCs with varying levels of OprD and MexAB-OprM/MexXY-OprM efflux pumps, PDC variants resistant to ceftolozane-tazobactam, and KPC, GES or VIM carbapenemases. Number of isolates at each MIC for cefepime relative to cefepime-tazobactam, cefepime-taniborbactam, ceftazidime-avibactam and ceftolozane-tazobactam with BLI fixed at 4 µg/mL in all cases except for FEP-TZB, where BLI was fixed at 8 µg/mL. MIC\textsubscript{50}/MIC\textsubscript{90} values were 32/≥256, 16/≥64, 4/8, 16/≥64 and 32/≥64 µg/mL for FEP, FEP-TZB, FEP-TAN, CZA and C/T respectively. %S, defined as MIC ≤ 8 µg/mL for FEP alone, FEP-TZB, FEP-TAN and CZA, or C/T with MIC ≤ 4 µg/mL were 22%, 29.3%, 92.7%, 46.3% and 31.7% respectively. FEP = cefepime, FEP-TZB = cefepime-tazobactam, FEP-TAN = cefepime-taniborbactam, CZA = ceftazidime-avibactam and C/T = ceftolozane-tazobactam.
Figure 6. Time-kill curves for ceftapime-taniborbactam relative to ceftazidime-avibactam in two metallo-β-lactamase producing clinical isolates. The log_{10} of the viable CFU/mL is displayed on the y-axis versus time (hours) on the x-axis.

| Test article          | K. pneumoniae CDC-0049 (NDM) | P. aeruginosa PS-12 (ST-235, VIM-2) |
|-----------------------|-----------------------------|-----------------------------------|
| Cefepime              | 256                         | 32                                |
| Cefepime-taniborbactam | 4                           | 4                                 |
| Ceftazidime-avibactam | >2,048                      | 64                                |

*Modal MIC values are reported. **MIC values reported for combinations are for the β-lactam with BLI at a static concentration of 4 µg/mL.
Curves for 1×, 2× and 4×MIC for cefepime with taniborbactam fixed at 4 µg/mL are shown for each strain while the curves for ceftazidime at 32 µg/mL with avibactam fixed at 4 µg/mL, representing 0.015×MIC (K. pneumoniae CDC-0049) and 0.5×MIC (P. aeruginosa PS-12) for ceftazidime, but well above the CLSI susceptibility breakpoint for ceftazidime-avibactam in Enterobacterales and P. aeruginosa of 8 µg/mL.