Ceftazidime-Avibactam in Combination With Fosfomycin: A Novel Therapeutic Strategy Against Multidrug-Resistant *Pseudomonas aeruginosa*

Krisztina M. Papp-Wallace,[1,4,5,6] Elise T. Zeiser,[1] Scott A. Becka,[1] Steven Park,[10] Brigid M. Wilson,[1,3,4] Marisa L. Winkler,[11] Roshan D'Souza,[12] Indresh Singh,[12] Granger Sutton,[12] Derrick E. Fouts,[12] Liang Chen,[10] Barry N. Kreiswirth,[10] Evelyn J. Ellis-Grosse,[13] George L. Drusano,[14] David S. Perlin,[10] and Robert A. Bonomo[1,2,3,4,5,6,7,8,9]

[1]Research Service, [2]Medical Service, and [3]Geriatric Research Education and Clinical Center, Louis Stokes Cleveland VA Medical Center, [4]Department of Medicine, [5]Department of Biochemistry, [6]Center for Proteomics and Bioinformatics, [7]Department of Molecular Biology and Microbiology, and [8]Department of Pharmacology, Case Western Reserve University (CWRU), and [9]CWRU–Cleveland VAMC Center for Antimicrobial Resistance and Epidemiology, Cleveland, Ohio; [10]Hackensack Meridian Health Center for Discovery and Innovation, Nutley, New Jersey; [11]Brigham and Women's Hospital, Boston, Massachusetts; [12]J. Craig Venter Institute, Rockville, Maryland; [13]Nabriva Therapeutics, King of Prussia, Pennsylvania; and [14]Institute for Therapeutic Innovation, University of Florida, Orlando

Previously, by targeting penicillin-binding protein 3, *Pseudomonas*-derived cephalosporinase (PDC), and MurA with ceftazidime-avibactam-fosfomycin, antimicrobial susceptibility was restored among multidrug-resistant (MDR) *Pseudomonas aeruginosa*. Herein, ceftazidime-avibactam-fosfomycin combination therapy against MDR *P. aeruginosa* clinical isolate CL232 was further evaluated. Checkerboard susceptibility analysis revealed synergy between ceftazidime-avibactam and fosfomycin. Accordingly, the resistance elements present and expressed in *P. aeruginosa* were analyzed using whole-genome sequencing and transcriptome profiling. Mutations in genes that are known to contribute to β-lactam resistance were identified. Moreover, expression of bla_{PDC}, the *mexAB-oprM* efflux pump, and *murA* were upregulated. When fosfomycin was administered alone, the frequency of mutations conferring resistance was high; however, coadministration of fosfomycin with ceftazidime-avibactam yielded a lower frequency of resistance mutations. In a murine infection model using a high bacterial burden, ceftazidime-avibactam-fosfomycin significantly reduced the *P. aeruginosa* colony-forming units (CFUs), by approximately 2 and 5 logs, compared with stasis and in the vehicle-treated control, respectively. Administration of ceftazidime-avibactam and fosfomycin separately significantly increased CFUs, by approximately 3 logs and 1 log, respectively, compared with the number at stasis, and only reduced CFUs by approximately 1 log and 2 logs, respectively, compared with the number in the vehicle-treated control. Thus, the combination of ceftazidime-avibactam-fosfomycin was superior to either drug alone. By employing a "mechanism-based approach" to combination chemotherapy, we show that ceftazidime-avibactam-fosfomycin has the potential to offer infected patients with high bacterial burdens a therapeutic hope against infection with MDR *P. aeruginosa* that lack metallo-β-lactamases.

Keywords. *Pseudomonas aeruginosa*; β-lactams; fosfomycin; combination therapy.

Pseudomonas aeruginosa causes nearly 10% of hospital-acquired infections and is the most prevalent gram-negative pathogen isolated from patients with ventilator-associated pneumonia [1, 2]. Additionally, *P. aeruginosa* is difficult to treat with currently available antibiotics. Cationic antimicrobial peptides (eg, colistin) are often the only agents that remain effective against multidrug-resistant (MDR) *P. aeruginosa* [3]. Therefore, new therapeutic strategies against MDR *P. aeruginosa* are essential.

Ceftazidime-avibactam was approved for clinical use for the treatment of urinary tract infections caused by Enterobacteriaceae. Avibactam is a novel β-lactamase inhibitor shown to be effective against the class A and C β-lactamases expressed in *P. aeruginosa* [4]. However, the activity of avibactam toward class D β-lactamases (ie, OXA-2, OXA-5/10, and OXA-50 [*poxB*] families) from *P. aeruginosa* is more limited [5–7]. Winkler and Papp-Wallace et al demonstrated that ceftazidime-avibactam possesses potent activity against MDR *P. aeruginosa*, raising optimism regarding its potential use in the treatment of infections [8]. However, 18% of the MDR *P. aeruginosa* clinical isolates collected from a decade ago were resistant to ceftazidime-avibactam (Table 1) [8]. This observation was consistent with data obtained from the International Network for Optimal Resistance Monitoring Program, in the United States [9]. Importantly, by combining ceftazidime-avibactam with fosfomycin and targeting penicillin-binding protein 3 (PBP-3), the β-lactamases (eg, *Pseudomonas*-derived cephalosporinase

Table 1. Minimum Inhibitory Concentrations of Various *Pseudomonas aeruginosa* Clinical Isolates

Strain	MEM	TZP	CIP	COL	CAZ	CAZ-AVI[a]	FOS	CAZ- AVI-FOS[a,b]	CAZ- AVI-COL[a,c]
PA01	0.5	1	1	0.5	2	1	64	1	0.5
CL232	64	>128	16	2	>32	32	32	4	32
715	32	>128	32	2	>32	32	8	8	32
716	64	16	64	2	>32	>32	128	64	4–8
776	64	128	64	2	>32	>32	128	4	2
795	32	>128	128	2	>32	>32	128	4	128
835	16	16	2	2	>32	32	64	4	8
839	16	128	>128	2	>32	>32	64	4	2–4
834	16	16	>128	2	>32	16	>128	4	8–16
851	32	128	64	0.25	>32	16	>128	8	<0.06

Data are mg/L and were modified from [8].

Abbreviations: AVI, avibactam; CIP, ciprofloxacin; COL, colistin; CAZ, ceftazidime; FOS, fosfomycin MEM, meropenem; TZP, piperacillin-tazobactam.

[a]AVI was maintained at 4 mg/L in combinations.

[b]FOS and CAZ were used at the same concentration.

[c]COL was added to all plates at 0.5 mg/L.

[PDC] also known as *Pseudomonas* AmpC), and MurA (a UDP-N-acetylglucosamine-1-carboxyvinyltransferase), susceptibility was restored in vitro to most ceftazidime-avibactam–resistant *P. aeruginosa* isolates in the collection (Figure 1 and Table 1) [8].

The oral formulation of fosfomycin is approved in the United States for the treatment of urinary tract infections caused by gram-positive and gram-negative bacteria, including *P.*

aeruginosa. Fosfomycin possesses bactericidal action against gram-negative bacteria, and an intravenous formulation is currently in development for US application and has completed phase I and II/III clinical trials (NCT02753946, NCT03709927, and NCT02178254 [10]). In some countries, intravenously administered fosfomycin is already used to treat a wide range of infections, including pneumonia [11, 12]. Interest in the
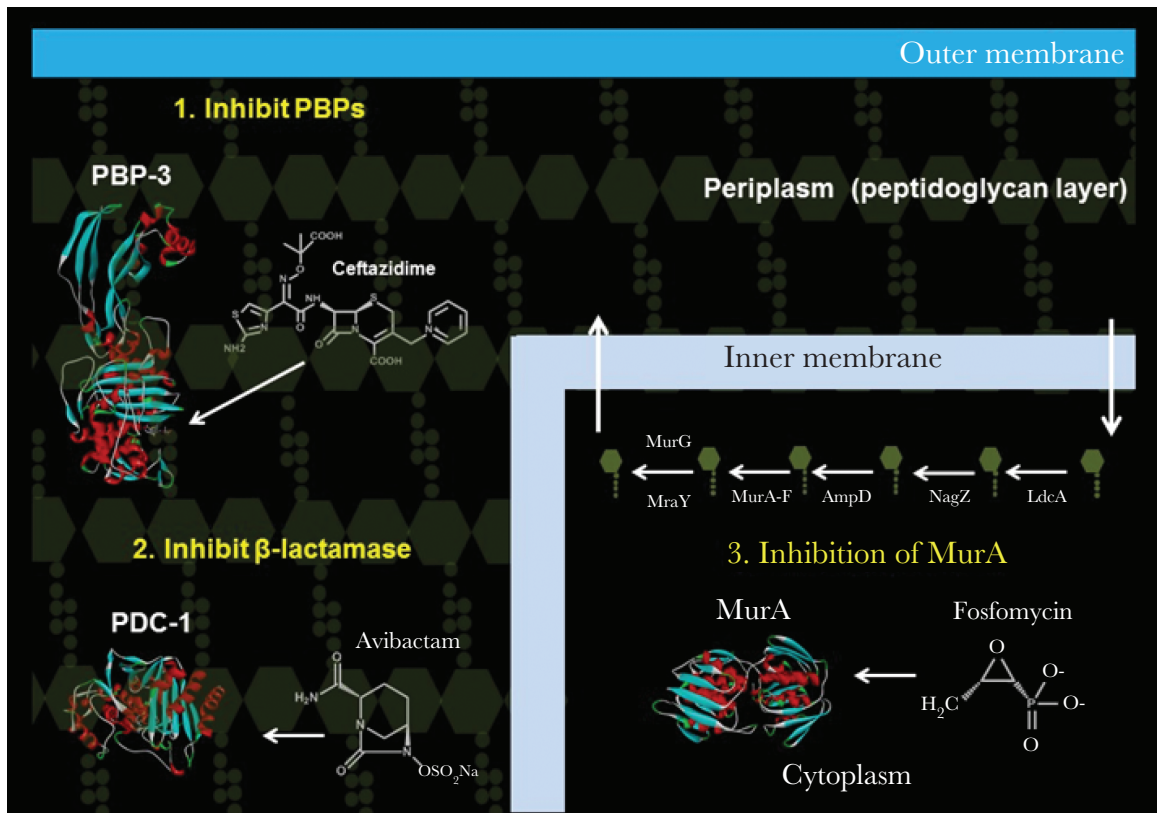

Figure 1. Structures of ceftazidime, avibactam, and fosfomycin and their targets, penicillin-binding protein 3 (PBP-3; Protein Data Bank identifier 3OCN), *Pseudomonas*-derived cephalosporinase 1 (PDC-1; 4HEF), and MurA (5BQ2), respectively.

possible use of fosfomycin in combination with β-lactams against infections due to MDR *P. aeruginosa* is increasing. Indeed, several recent studies revealed synergism between fosfomycin and β-lactams (ie, carbapenems and ceftolozane-tazobactam) [13–19].

The decision to combine ceftazidime-avibactam with fosfomycin was based on the rationale that even though fosfomycin can downregulate expression of PBP-3 and induce PDC expression, avibactam is so potent that it can significantly hinder the hydrolytic activity of PDC, as well as other class A and C β-lactamases present in *P. aeruginosa* [5, 8, 20]. Notably, many MDR *P. aeruginosa* already possess a derepressed bla_{PDC} [8]. With avibactam targeting the class A and C β-lactamases, ceftazidime and fosfomycin are free to disrupt the cell wall recycling pathway, using 2 different strategies to kill the bacteria. One limitation of ceftazidime-avibactam-fosfomycin is the lack of coverage against *P. aeruginosa* producing metallo-β-lactamases; fortunately, the prevalence of metallo-β-lactamase–producing *P. aeruginosa* in United States remains low [21, 22].

Here, we determined the activity of ceftazidime-avibactam combined with fosfomycin against *P. aeruginosa* strain CL232, which was chosen because of its MDR phenotype and limited susceptibility profile to currently available agents (ie, colistin). In addition, the resistance determinants present and expressed in *P. aeruginosa* CL232 were identified. The mutation frequency of the single agents and the combinations were tested using this isolate. Finally, the in vivo activity of ceftazidime-avibactam combined with fosfomycin was assessed in a neutropenic mouse thigh infection model, using a high bacterial burden of *P. aeruginosa* CL232.

METHODS

Critical Reagents

P. aeruginosa stains PA01 and CL232 were used in this study [8]. For in vitro assays, ceftazidime and fosfomycin were purchased from Sigma-Aldrich, pharmaceutical-grade ceftazidime-avibactam was also used, and avibactam was provided by Allergan. For in vivo experiments, pharmaceutical-grade ceftazidime was used, while fosfomycin and avibactam were obtained from Zavante and Allergan, respectively.

Growth Curve Analysis

P. aeruginosa strains were grown in Mueller-Hinton (MH) broth with or without ceftazidime-avibactam (16 μg/mL–4 μg/mL), fosfomycin (16 μg/mL), or ceftazidime-avibactam-fosfomycin (16 μg/mL–4 μg/mL–16 μg/mL) at 37°C with shaking for 11 hours. At selected time points, the OD_{600} was measured.

Checkerboard Analysis

The minimum inhibitory concentrations (MICs) were determined for the combinations, and a fractional inhibitory concentration (FIC) index was calculated using checkerboard analysis in MH broth as previously described for *P. aeruginosa* CL232 [23].

Whole-Genome Sequencing (WGS)

Genomic DNA was purified from *P. aeruginosa* PA01, *P. aeruginosa* CL232, and its fosfomycin-resistant mutant (CL232FR) recovered from treated mice, using the MasterPure gram-positive DNA purification kit as recommended by the manufacturer. The genome was sequenced by Illumina NextSeq 500 Mid Output Kit or HiSeq X with 300 cycles (2 × 150 bp). Paired-end libraries were constructed using Illumina NexteraXT kits. Sequence reads were generated with a target average read depth of approximately 100-fold coverage. Sequence reads for each isolate were assembled individually, using Velvet v. 1.2.07 [24], and annotated using the National Center for Biotechnology Information's Prokaryotic Genome Annotation Pipeline [25]. Single-nucleotide polymorphisms and insertion and deletion calling was performed using BWA, Samtools, and Vcftools, followed by annotation using SnpEff [26–29]. Raw DNA sequence reads were submitted to the National Center for Biotechnology Information Sequence Read Archive, and annotated genomes were deposited in the GenBank WGS repository (BioSample identifiers SAMN05774260 and SAMN10485642 and GenBank accession number MPVE00000000).

RNA Sequencing (RNAseq)

P. aeruginosa PA01 and CL232 were grown in lysogeny broth at 37°C until an OD_{600nm} of 0.6 was reached. One milliliter of cells in log-phase growth was collected, mixed with 500 μL of RNAprotect Bacteria Reagent, and incubated at room temperature for 15 minutes. The RNeasy Mini Kit protocol was followed to purify the RNA from the samples. An on-column DNAse digestion using the RNAse-Free DNAse Set was conducted. In addition, a second step to remove residual DNA was conducted using the Turbo DNA-free kit protocol. RNAseq and data analysis were conducted by the Genomics Core facility and the Institute for Computational Biology Core, respectively, at Case Western Reserve University, including quality control of the submitted RNA specimens; library preparation, using the Illumina ScriptSeq Complete Gold (Bacteria) kit; quality control of the library preparation; use of the Illumina HiSeq 2500 Rapid Run flow cell system (2 × 75 bp); generation of the reads per kilobase million data set, including assessment of the statistical significance of gene expression changes between *P. aeruginosa* PA01 and CL232; and cluster of orthologous groups analysis. The RNAseq data set and cluster of orthologous groups analysis are presented in Supplementary Table 1 and Supplementary Figure 1, respectively.

Determination of Mutation Frequency

To determine the mutation frequency in *P. aeruginosa* CL232 after exposure to ceftazidime-avibactam, fosfomycin, and ceftazidime-avibactam-fosfomycin, *P. aeruginosa* CL232 was grown in MH broth and concentrated to a high inoculum (10^{10}

colony-forming units [CFU]/mL). Cells were plated onto MH agar plates containing drug concentrations 4 times greater than the MIC and on MH agar only, to enumerate the total number of viable cells. The mutation frequency was calculated by dividing the number of cells on the drug-containing plates by the number of cells on the control plates.

Mice

Outbred 6–8-week-old female CD-1 mice were purchased from Charles River Laboratories. Mice were cared for according to the Institutional Animal Care and Use Committee guidelines of Rutgers University. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animal experiments and was adherent to principles stated in the National Research Council's 1996 *Guide for the Care and Use of Laboratory Animals*. The facilities where this research was conducted are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Fosfomycin Tolerance

As the humanized doses of fosfomycin were very high, a tolerance study was conducted in both immunocompetent and neutropenic mice. The dosing regimen for fosfomycin consisted of 6 doses administered subcutaneously at 0, 3, 8, 11, 16, and 19 hours at alternating doses of 70.4 mg and 46.9 mg. Outbred 6–7-week-old female CD-1 mice weighing 23–26 g were used with the dosing regimen stated above (adjusted for a body weight of 25 g). For neutropenic mice, a subset of CD-1 mice were administered 150 mg/kg and 100 mg/kg of cyclophosphamide via intraperitoneal injection 4 days and 1 day, respectively, before the administration of fosfomycin.

Neutropenic Thigh Infection Model and Bacterial Burden

In all studies, recumbent animals were considered moribund and humanely euthanized. Female outbred 6–8-week-old CD-1 mice (18 per treatment condition) were rendered neutropenic as described above and challenged with 2.0×10^7 CFU/thigh of *P. aeruginosa* CL232. The mice were treated with either vehicle (water for injection) or the following dosing regimen. A single 60/40 split dose was administered. One hour after infection, 9.54 mg of ceftazidime and/or 3.11 mg of avibactam and/or 70.4 mg of fosfomycin was given, while 4 hours after infection, 6.36 mg of ceftazidime and/or 2.08 mg of avibactam and/or 46.9 mg of fosfomycin was given. For measurement of bacterial burden, mice were euthanized by carbon dioxide narcosis at the end point of 24 hours after initiating infection. The thighs were harvested, and tissue homogenates were serially diluted in sterile phosphate-buffered saline, plated on MH agar, and incubated at 37°C for up to 48 hours. CFUs were counted. To screen for development of drug resistance, selected colonies from the groups that received ceftazidime-avibactam or fosfomycin only for 24 hours were replated on MH agar containing 192 and 12 μg/mL ceftazidime-avibactam or 192 μg/mL fosfomycin, respectively, and incubated at 37°C for up to 48 hours. The concentrations used are approximately 3 times the MIC for the original *P. aeruginosa* CL232 strain used.

Statistical Analysis

For neutropenic thigh infection models, mean log CFUs/thigh were compared across treatment groups and to stasis, using analysis of variance and Tukey post hoc tests, with adjusted P values calculated to assess differences among treatment pairs. All analyses were performed in R, version 3.5.0 [29, 30].

RESULTS AND DISCUSSION

In Vitro Efficacy of Ceftazidime-Avibactam-Fosfomycin

Previous analysis found that 9 out of 50 strains in a panel of archived clinical isolates of *P. aeruginosa* tested resistant to ceftazidime-avibactam (Table 1) [8]. The addition of fosfomycin to the ceftazidime-avibactam combination restored susceptibility to approximately 90% of the isolates (MIC range,
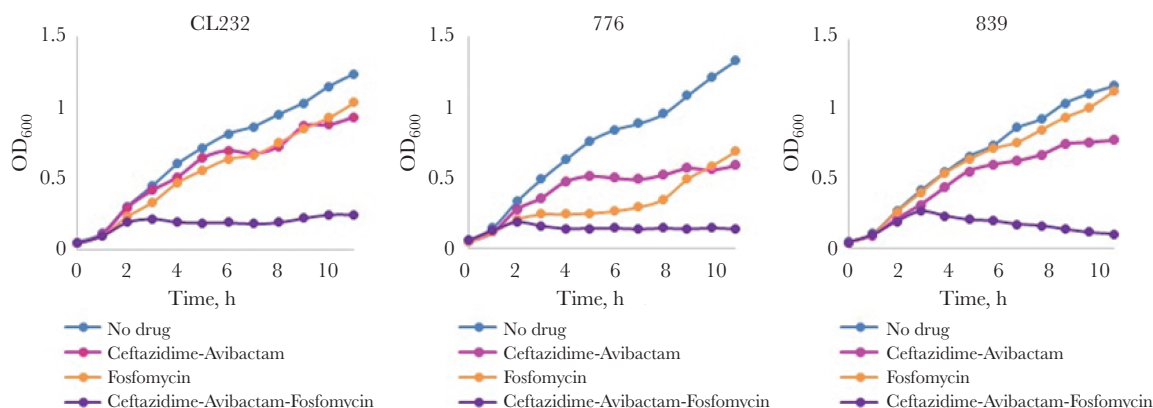

Figure 2. Growth of *Pseudomonas aeruginosa* strains CL232, 776, and 839 in Mueller-Hinton broth with or without ceftazidime-avibactam (16 and 4 μg/mL, respectively), fosfomycin (16 μg/mL), or ceftazidime-avibactam-fosfomycin (16, 4, and 16 μg/mL, respectively) over 11 hours.

4–8 µg/mL) with the exception of strain 716 (MIC, 64 µg/mL). However, isolate 716 was susceptible to piperacillin-tazobactam (MICs, 16 and 4 µg/mL, respectively).

In this study, growth curve analysis was completed with ceftazidime-avibactam–resistant *P. aeruginosa* strains CL232, 776, and 839, using ceftazidime-avibactam, fosfomycin, ceftazidime-avibactam-fosfomycin and a no drug control (Figure 2). The presence of ceftazidime-avibactam-fosfomycin in the medium prevented the growth of all MDR *P. aeruginosa* strains for up to 11 hours, compared with treatment with ceftazidime-avibactam and fosfomycin separately. Based on the consistent growth pattern obtained for the strain, *P. aeruginosa* CL232 was selected for subsequent in vitro and in vivo studies. *P. aeruginosa* CL232 is highly resistant to all β-lactams and β-lactam–β-lactamase inhibitor combinations, as well as to fluoroquinolones, fosfomycin, and tigecycline (Table 1) [8].

Synergy of the Ceftazidime-Avibactam-Fosfomycin Combination
To assess whether ceftazidime-avibactam and fosfomycin were synergistic, a checkerboard analysis was conducted using *P. aeruginosa* CL232. The MICs were determined for the combinations, and a fractional inhibitory concentration (FIC) index was calculated. An FIC index of ≤0.5 was obtained for one of the combinations tested (Table 2). The combination of 25 µg/mL and 6.25 µg/mL of ceftazidime and avibactam, respectively, combined with 12.5 µg/mL of fosfomycin possessed an FIC index of 0.5. The addition of a β-lactamase inhibitor, avibactam, reversed the antagonism that was previously reported with fosfomycin and β-lactams [20]. Our data showed that β-lactamase inhibition is a vital component of this combination.

Resistome of *P. aeruginosa* CL232
To identify which determinants are contributing to the ceftazidime-avibactam and fosfomycin resistance phenotypes, the genome of *P. aeruginosa* CL232 was analyzed and compared to the *P. aeruginosa* PA01 genome. The list of genes involved in β-lactam and fosfomycin resistance in *P. aeruginosa* is displayed in Table 3 [31, 32].

The PBPs in *P. aeruginosa* CL232 were examined, and single amino acid substitutions were found in PBP1B, PBP3A, and PBP4; the contribution of these changes to β-lactam resistance remains to be determined. Nevertheless, knock out of the gene encoding PBP4 (*dacB*) was shown to increase resistance to β-lactams [33]. Importantly, *ftsI*, which encodes PBP-3, the target of ceftazidime, was unaltered in *P. aeruginosa* CL232. Moreover, *P. aeruginosa* CL232 possesses 2 chromosomal β-lactamase genes, bla_{PDC-34} and $bla_{OXA-488}$, of which the latter is a bla_{OXA-50} family member (Table 3). The expression of bla_{PDC-34} and $bla_{OXA-488}$ is linked to cell wall recycling and is induced by exposure to certain β-lactams [33–35]. PDC-34, the dominant β-lactamase in *P. aeruginosa* CL232, possessed several amino acid substitutions, including G1D, A29T, T79A, Q128R, V178L, and G364A, which are not located within the active site motifs of AmpCs [36]. Thus, these changes likely do not result in an expanded-spectrum phenotype. β-lactamases in the OXA-50 family play a minor role in β-lactam resistance and, thus, are unlikely to significantly influence the resistance phenotype of this strain [35]. AmpR is the transcriptional regulator that positively regulates bla_{PDC-34} expression, but it negatively regulates $bla_{OXA-488}$ expression [37]. *ampR* in *P. aeruginosa* CL232 carries a mutation that corresponds to an amino acid substitution at position D135, which was previously shown to lead to overexpression of bla_{PDC} (Table 3) [38]. Additionally, a frameshift mutation was also identified in *oprD* of *P. aeruginosa* CL232; OprD is the major porin for the entry of carbapenems into *P. aeruginosa* (Table 3). The mutation in *pbps* and derepressed bla_{PDC}, as well as the mutation in *oprD*, likely impact resistance to β-lactams in *P. aeruginosa* CL232 [39].

Alterations in 4 different resistance determinants (*murA*, *glpT*, *oprO*, and *oprP*) in *P. aeruginosa* can influence susceptibly to fosfomycin. *murA*, which encodes MurA, the target of fosfomycin, was unaltered in *P. aeruginosa* CL232 (Table 3). The entry of fosfomycin into the periplasmic space of *P. aeruginosa* is not well understood, and to date, 3 proteins, GlpT, a glycerol-3-phosphate transporter, and the OprO and OprP porins have

Table 2. Results of Checkerboard Analyses of Ceftazidime-Avibactam-Fosfomycin (CAZ-AVI-FOS) in Mueller-Hinton Broth, Using *Pseudomonas aeruginosa* CL232

Variable	CAZ-AVI MIC, mg/L	FOS MIC, mg/L	FIC CAZ-AVI	FOS FIC	FIC Index	Result
Alone	50–12.5	400				
Combined	12.5–3.12	800	0.25	2	2.3	No interaction
Combined	25–6.25	400	0.5	1	1.5	No interaction
Combined	25–6.25	200	0.5	0.5	1	No interaction
Combined	25–6.25	100	0.5	0.25	0.8	No interaction
Combined	25–6.25	50	0.5	0.13	0.6	No interaction
Combined	25–6.25	25	0.5	0.06	0.6	No interaction
Combined	25–6.25	12.5	0.5	0.03	0.5	Synergy
Combined	50–12.5	6.25	1	0.02	1.0	No interaction

Synergy is defined by an FIC index ≤ 0.5

Abbreviations: FIC, fractional inhibitory concentration; MIC, minimum inhibitory concentration.

Gene, Function, Mutation(s)/Substitution(s)	Log$_2$ Fold Change
mexABoprM	
β-lactam RND transporter	
MexA substitutions: none	+1.7
MexB substitutions: none	+3.1
OprM substitutions: none	+2.6
mexXYoprM	
β-lactam RND transporter	
MexX substitutions: A30T	No change
MexY substitutions: I536V, T543A, G589A, Q840E, N1036T, Q1039R	No change
OprM substitutions: none	+2.6
mexCDoprJ	
β-lactam RND transporter	
MexC substitutions: P47S, R76Q, H310R, S330A, A378P, P383S, A384Y	No change
MexD substitutions: T87S, A155T, E257Q, V660I, N669D, S685G, I701V, S845A, S915A, I982V, K1031R, S1040T	No change
OprJ substitutions: D68G, M69V	No change
oprD	
Porin	
Mutation: frameshift	−2.2
oprF	
Porin	
Substitutions: none	No change
mrcB (ponB, pbpF)	
PBP1b: transglycolyase-transpeptidase	
Substitutions: V185A, Y568H, T911A	No change
pbpA	
PBP2: transglycolyase-transpeptidase	
Substitutions: none	No change
ftsI	
PBP3: transpeptidase	
Substitutions: none	No change
pbpC	
PBP3A	
Substitutions: A104P	No change
dacB	
PBP4: D-alanyl-D-alanine carboxypeptidase	
Substitutions: Q156H	No change
dacC	
PBP5: D-alanyl-D-alanine carboxypeptidase	
Substitutions: none	No change
pbpG	
PBP7 D-alanyl-D-alanine endopeptidase	
Substitutions: none	No change
bla$_{OXA-488}$ *(poxB)*	
Class D β-lactamase	
Substitutions: T16$_L$A, Q29R	No change
bla$_{PDC-34}$	
Class C β-lactamase	
G1D, A29T, T79A, Q129R, V179L, G363A	+6.6
ampR	
Transcriptional regulator of *bla*$_{PDC}$	
Substitutions: E114A, D135A, G283E, M288R	No change
fosA	
Glutathione transferase: enzymatically inactivates fosfomycin	
Substitutions: none	Not present in RNA sequencing data set
glpT	
Glycerol-3-phosphate transporter: used for entry of fosfomycin	
Substitutions: none	No change
oprO	
Porin: used for entry of fosfomycin	
Substitutions: none	No change
oprP	
Porin: used for entry of fosfomycin	
Substitutions: A98V	No change
murA	
UDP-N-acetylglucosamine 1-carboxyvinyltransferase	
Substitutions: none	+2.3

been shown to contribute to drug entry [40, 41]. In *P. aeruginosa* CL232, only *oprP* possessed a mutation that resulted in an A98V amino acid change; however, the contribution of this substitution on fosfomycin entry remains unknown (Table 3).

To evaluate which resistance determinants are expressed by *P. aeruginosa* CL232, RNAseq experiments were conducted on *P. aeruginosa* CL232, and findings were compared to those for *P. aeruginosa* PA01 (Table 3). Results of transcriptome analysis was consistent with the predictions gleaned from WGS data, because *mexABoprM* and *bla*$_{PDC-34}$ were upregulated (an immunoblot conducted previously confirmed upregulation of *bla*$_{PDC-34}$ [8]) and *oprD* was downregulated (Table 3). These 3 modifications likely contribute to β-lactam resistance in *P. aeruginosa* CL232. Analysis of the upstream promoter of *mexABoprM* revealed that mutations were not present between *mexA* and *mexR*, the gene encoding the transcriptional regulator of the *mexABoprM* operon. Expression of *murA*, which encodes the target for fosfomycin, was also upregulated, while *fosA*, encoding a glutathione-S-transferase known to inactivate fosfomycin, was not expressed by either strain (Table 3) [42]. One could hypothesize that increased expression of *murA* could lead to fosfomycin resistance, because more MurA would be present in the cytoplasm and, thus, higher concentrations of fosfomycin would be required to inhibit the enzyme.

Ceftazidime-Avibactam Decreases the Mutation Frequency of Fosfomycin Alone In Vitro

To assess the contribution of the evolution of mutations in *P. aeruginosa* CL232 to the development of resistance to ceftazidime-avibactam, fosfomycin, and ceftazidime-avibactam-fosfomycin, mutation frequencies were determined. When *P. aeruginosa* CL232

Table 4. Mutation Frequency for *Pseudomonas aeruginosa* CL232 After Exposure to Fosfomycin (FOS), Ceftazidime-Avibactam (CAZ-AVI), and CAZ-AVI-FOS

Condition	Mutation Frequency	MIC(s),[a] mg/L
FOS	1.9×10^{-5}	64
CAZ-AVI	9.1×10^{-9}	64 and 4, respectively
CAZ-AVI-FOS	9.1×10^{-9}	16, 4, and 16, respectively

Abbreviation: MIC, minimum inhibitory concentration.
[a]Agar dilution.

was plated on agar containing fosfomycin at 4 times the MIC, a mutation frequency of 1.9×10^{-5} was obtained (Table 4). When *P. aeruginosa* CL232 was grown on 4 times the MIC of ceftazidime-avibactam, minor growth was observed, corresponding to a mutation frequency of 9.1×10^{-9} (Table 4). Finally, the cells plated on the ceftazidime-avibactam-fosfomycin combination demonstrated the same mutation frequency as those on the ceftazidime-avibactam combination (Table 4). Our data suggest that ceftazidime-avibactam diminished the selection of fosfomycin-resistant mutants of *P. aeruginosa* CL232 by >4-log orders.

High Doses of Fosfomycin Causes Injection Site Side Effects in Neutropenic Mice

Because the humanized doses of fosfomycin were very high, a tolerance study was conducted in immunocompetent and neutropenic mice. Adverse reactions were not observed in immunocompetent animals during the drug administrations,

and mice were alert and responsive until the study end date (5 days after administration of the study drugs). However, in cyclophosphamide-induced neutropenic mice that were administered humanized doses of fosfomycin alone or in combination exhibited signs of drug toxicity after the fourth dose. Three mice (30%) became severely moribund and were humanely euthanized. The euthanized mice were necropsied, and gross evaluation showed mild inflammation of the internal organs; however, the injection sites had severe edema-like structures that were filled with fluid. The disodium salt composition of the fosfomycin, in combination with the antiinflammatory effect of cyclophosphamide, were likely important in contributing to these fluid-filled masses in the 3 euthanized mice. The remaining mice had ruffled fur and were slightly lethargic. Irritation was observed in 4 of 7 mice at the site of injection (the scruff). The mice were fully recovered at 24 hours and remained bright and alert for the next 4 days.

Despite the toxicity observed with fosfomycin in the neutropenic mouse model, fosfomycin is often used in combination with a variety of drugs, including β-lactams, for treatment of infections. In a meta-analysis of the clinical literature, the safety of fosfomycin was assessed alone and in combination [43]. Moreover, the results of the ZEUS clinical trial (clinical trials registration NCT02753946) were aligned with what has been observed outside the United States, where there is a long history of fosfomycin at dosages up to 24 g/day [44]. However, since intravenous fosfomycin contains a high salt load inherent to the
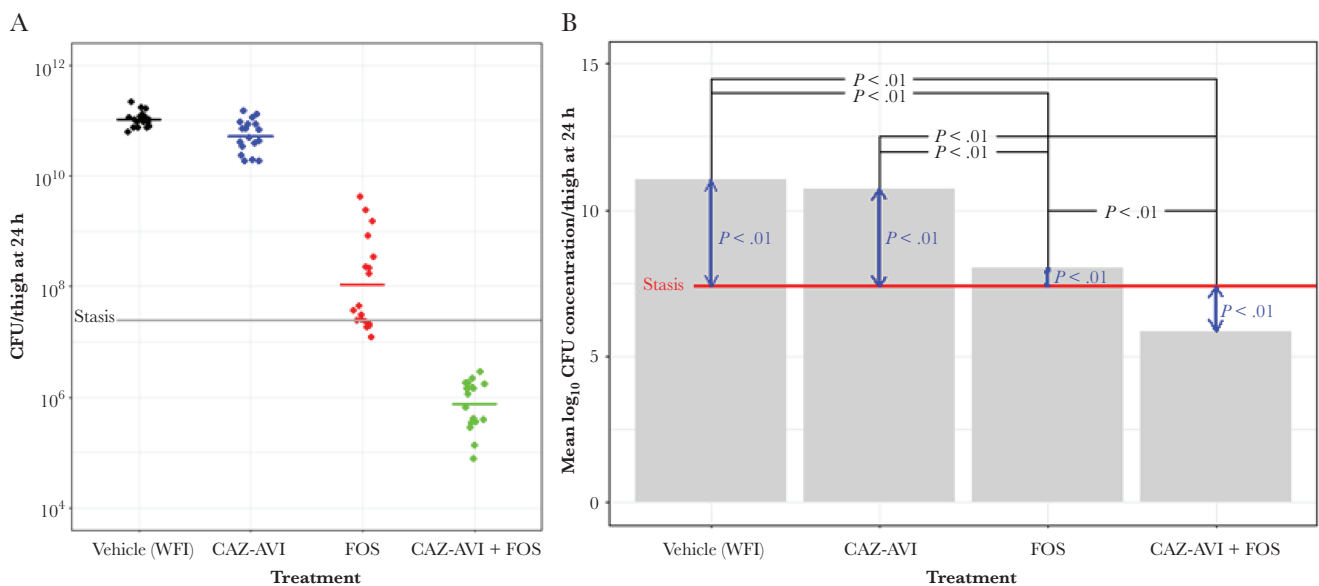

Figure 3. CD-1 mice (18 mice per treatment condition) were rendered neutropenic and challenged with 2.0×10^7 *Pseudomonas aeruginosa* CL232 colony-forming units (CFUs)/thigh. The mice were treated with either vehicle (water for injection [WFI]) or the following dosing regimen. A single 60/40 split dose was administered subcutaneously. One hour after infection, 9.54 mg of ceftazidime (CAZ) and/or 3.11 mg of avibactam (AVI) and/or 70.4 mg of fosfomycin (FOS) were administered, whereas 4 hours after infection, 6.36 mg of CAZ and/or 2.08 mg of AVI and/or 46.9 mg of FOS was given. *A*, CFUs/thigh for each mouse at an end point of 24 hours is presented for all treatment conditions; stasis is represented by the gray line. *B*, Mean $\log_{10}$ CFU concentration/thigh at an end point of 24 hours after initiating infection is represented. The red line represents stasis. In black are the statistical differences (expressed as *P* values) between treatment conditions, and in blue are the statistical differences (expressed as *P* values) between stasis and the treatment conditions.

disodium formulation, each gram contains 330 mg of sodium; thus, hypokalemia may be a concern in some patients.

Ceftazidime-Avibactam-Fosfomycin Decreases the Bacterial Burden in a Neutropenic Murine Thigh Infection Model

CD-1 mice were rendered neutropenic and challenged with 2.0×10^7 colony-forming units (CFU)/thigh of *P. aeruginosa* CL232. The mice were treated with vehicle, ceftazidime-avibactam, fosfomycin, or ceftazidime-avibactam-fosfomycin, using a single 60/40 split dose, 1 hour after infection. Human pharmacokinetics values were used to perform a 5000-iteration Monte Carlo simulation. The schedules, dosing amounts, and split were chosen to humanize the exposures. The murine peaks were calculated to not exceed the upper 95% confidence bound from the human-data Monte Carlo simulation. The results in Figure 3A show the end point at 24 hours as compared to stasis. This analysis revealed that ceftazidime-avibactam-fosfomycin was the most effective combination, with CFUs/thigh in the 10^{5-6} range, compared with 10^{11} for the vehicle-treated mice. Specifically, the ceftazidime-avibactam-fosfomycin combination was found to significantly reduce CFUs by approximately 2 logs below those at stasis (Figure 3B). Ceftazidime-avibactam and fosfomycin dosed separately increased CFUs from those at stasis by approximately 3 logs and 1 log, respectively (Figure 3B). Among paired comparisons of treatments to each other and to stasis, all combinations except vehicle compared to the ceftazidime-avibactam treatment arm differed significantly (adjusted $P < .01$).

Given the minor impact of ceftazidime-avibactam alone and fosfomycin alone in the mouse model, the bacteria isolated from the mouse thighs were selected on plates containing each drug, to assess for potential resistance acquired in vivo. In mice treated with ceftazidime-avibactam, observed regrowth of the *P. aeruginosa* CL232 strain was likely due to insufficient drug exposure, because the bacteria were not resistant to ceftazidime-avibactam. Conversely, the isolates obtained from the mice treated with fosfomycin alone were determined to be resistant; the fosfomycin MIC increased from 64 mg/L to 2048 mg/L. The fosfomycin-resistant isolate was subjected to WGS, and a single nucleotide change (C228A) was identified that resulted in the introduction of a stop codon in *glpT*, which encodes a glycerol-3-phosphate transporter that is used by fosfomycin for entry into *P. aeruginosa* [41]. Thus, in vivo, *P. aeruginosa* CL232 acquired resistance to fosfomycin. Elimination of fosfomycin may be involved in the acquisition of fosfomycin resistance.

Why Not Other Combinations of β-Lactam–β-Lactamase Inhibitor–Fosfomycin?

Ceftolozane-tazobactam-fosfomycin and aztreonam-avibactam-fosfomycin were also considered for testing. Indeed, ceftolozane-tazobactam-fosfomycin was previously found to be synergistic against MDR *P. aeruginosa* via time-kill analysis [17]. However, a major caveat of the ceftolozane-tazobactam-fosfomycin combination is that tazobactam is not as potent of a β-lactamase inhibitor as avibactam and cannot target PDC or class A carbapenemases [4]. In addition, several analyses have described PDC variants that are able to robustly hydrolyze ceftolozane, further diminishing interest in this pairing [45–49]. The aztreonam-avibactam-fosfomycin combination possesses other liabilities. Even though avibactam can target the class A and C β-lactamases and aztreonam would extend coverage to class B metallo-β-lactamases, the aztreonam-avibactam combination is not very effective in vitro (90% MIC, 32 µg/mL) against MDR *P. aeruginosa* as compared to carbapenem-resistant *Enterobacteriaceae* (90% MIC, 1 µg/mL) [50].

In conclusion, despite the introduction of highly effective therapies, MDR *P. aeruginosa* is still a significant threat to vulnerable and immunocompromised patients. Here, using a "mechanism-based" approach targeting important cell wall enzymes and resistance determinants, we showed that MDR *P. aeruginosa* can be readily overcome. In a high-bacterial-burden infection model, we showed that CFUs were reduced by approximately 2 logs, relative to CFUs at stasis. These findings have significant implications for further studies directed at clinical applications. Such an approach may be beneficial even when treating more-susceptible *P. aeruginosa*. By lowering CFUs and mutation frequency, we hope to eradicate the infection.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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