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Unexpected Challenges in Treating Multidrug-Resistant Gram-Negative Bacteria: Resistance to Ceftazidime-Avibactam in Archived Isolates of *Pseudomonas aeruginosa*

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***Pseudomonas aeruginosa* is a notoriously difficult-to-treat pathogen that is a common cause of severe nosocomial infections. Investigating a collection of β-lactam-resistant *P. aeruginosa* clinical isolates from a decade ago, we uncovered resistance to ceftazidime-avibactam, a novel β-lactam/β-lactamase inhibitor combination. The isolates were systematically analyzed through a variety of genetic, biochemical, genomic, and microbiological methods to understand how resistance manifests to a unique drug combination that is not yet clinically released. We discovered that avibactam was able to inactivate different AmpC β-lactamase enzymes and that bla_{PDC} regulatory elements and penicillin-binding protein differences did not contribute in a major way to resistance. By using carefully selected combinations of antimicrobial agents, we deduced that the greatest barrier to ceftazidime-avibactam is membrane permeability and drug efflux. To overcome the constellation of resistance determinants, we show that a combination of antimicrobial agents (ceftazidime/avibactam/fosfomycin) targeting multiple cell wall synthetic pathways can restore susceptibility. In *P. aeruginosa*, efflux, as a general mechanism of resistance, may pose the greatest challenge to future antibiotic development. Our unexpected findings create concern that even the development of antimicrobial agents targeted for the treatment of multidrug-resistant bacteria may encounter clinically important resistance. Antibiotic therapy in the future must consider these factors.**

The World Health Organization, Centers for Disease Control (CDC), and the Infectious Disease Society of America have sounded a worldwide "call to arms" on the subject of multidrug-resistant (MDR) Gram-negative bacteria (1–3). In addition to the increasing number of MDR bacteria plaguing health care systems, there is a "discovery void" of new antibiotics to treat bacterial infections (1). In the United States alone, at least 2 million people acquire infections with bacteria resistant to one or more classes of antibiotics, and 23,000 people die from drug-resistant infections (2). Not only do drug-resistant infections lead to significant loss of life and long-term complications among those they infect, but a major financial cost has also been attributed to antibiotic resistance. Such infections lead to increased utilization of valuable health care resources, more expensive or longer treatment, protracted hospital stays, and increased disability and death, leading to an estimate of the overall cost being up to $35 billion each year (2). The two strategies identified by the CDC for reducing the number of new antibiotic-resistant infections are (i) to improve the current antibiotic supply and (ii) to develop new antibiotics for treating resistant bacteria (2).

β-Lactam antibiotics (penicillins, cephalosporins, carbapenems, and monobactams) have been the mainstay of treatment for Gram-negative bacterial infections since the discovery of penicillin in the 1940s (4, 5). Resistance to β-lactams poses a significant threat to the continued successful treatment of both common and opportunistic pathogens (6). Resistant pathogens evolve a variety of features, including changes in the drug targets (the penicillin-binding proteins of the bacterial cell wall), impaired permeability (including increased mucosity, the absence of porins, and/or the presence of efflux pumps), and the presence of enzymes with the ability to inactivate the antibiotics (i.e., β-lactamase enzymes) (5, 6). Importantly, β-lactamase enzymes are evolving to confer resistance to all classes of β-lactam antibiotics (e.g., KPC-2, CTX-M-15, OXA, and AmpC enzymes) (5). An approach to combat infections by bacteria containing such enzymes is the development of β-lactamase inhibitors (BLIs), which act to cripple the catalytic capacity of the enzyme (Fig. 1A) (7). Regrettably, new β-lactamase variants are being discovered and inhibitor-resistant β-lactamases are appearing, necessitating the development of novel tactics (7).

Avibactam is one such novel non-β-lactam BLI which has been shown to be effective against class A, class C, and some class D β-lactamase enzymes (inactivation scheme in Fig. 1B) (8–11). Avibactam is currently being evaluated in combination with ceftazidime (ceftazidime-avibactam) in phase III clinical trials for its effectiveness against bacterial infections (NCT01644643, NCT01499290, NCT01808092, NCT01599806, NCT01595438, NCT01726023, and NCT01500239).

Received 29 August 2014 **Returned for modification** 6 October 2014
Accepted 22 November 2014

Accepted manuscript posted online 1 December 2014

Citation Winkler ML, Papp-Wallace KM, Hujer AM, Domitrovic TN, Hujer KM, Hurless KN, Tuohy M, Hall G, Bonomo RA. 2015. Unexpected challenges in treating multidrug-resistant gram-negative bacteria: resistance to ceftazidime-avibactam in archived isolates of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 59:1020–1029. doi:10.1128/AAC.04238-14.

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/AAC.04238-14.

doi:10.1128/AAC.04238-14

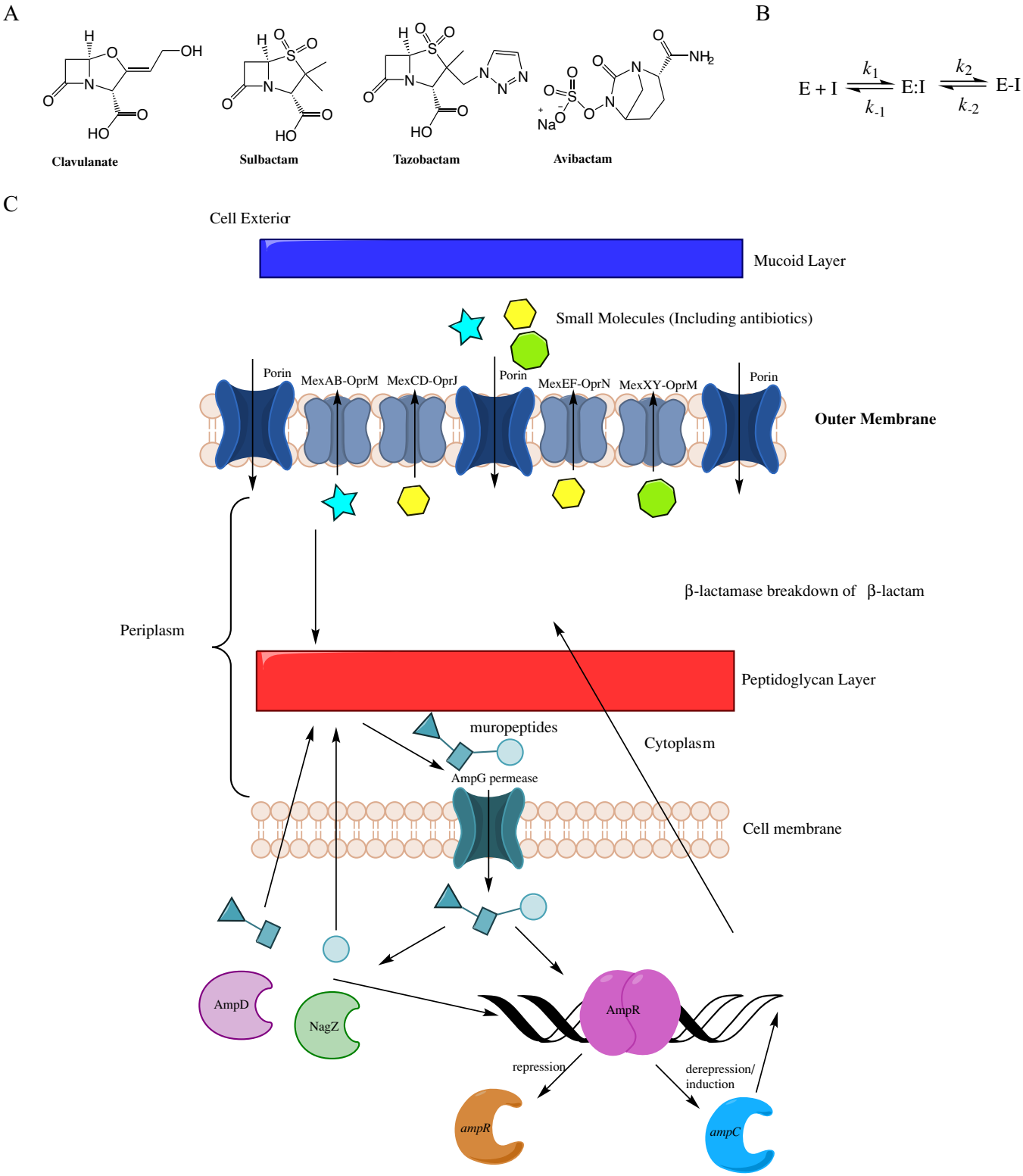


FIG 1 (A) BLIs. (B) Scheme of interaction of avibactam (I) with a β-lactamase (E) showing formation of the Michaelis-Menten complex (E:I), the formation of the acyl-enzyme (E-I), and recyclization of avibactam to regenerate active compound and allow repetition of the reaction. (C) Known resistance mechanisms in *P. aeruginosa*, including a mucoid layer, outer membrane porins, efflux pumps, PBP alterations, and β-lactamase upregulation. *P. aeruginosa* has a mucoid layer outside the outer membrane; increased thickness of this layer can lead to antibiotic resistance. Antibiotics enter the cell through porins in the outer membrane. Loss of these porins can lead to antibiotic resistance. *P. aeruginosa* also can carry efflux pumps in the outer membrane (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM); when these structures are present, antibiotics can be pumped out of the cell, leading to resistance. β-Lactamases can be present in the periplasmic space of the bacteria, which are able to break down β-lactam antibiotics and/or BLIs, leading to resistance to these compounds. The regulation of the chromosomal AmpC in *P. aeruginosa* is illustrated, which involves a complex relationship between peptidoglycan breakdown, β-lactam exposure, and gene regulation leading to overexpression of the AmpC enzyme. Lastly, the PBPs in the peptidoglycan layer can be altered to prevent interaction of β-lactam antibiotics with their targets.

An important potential use of avibactam is in the treatment of *Pseudomonas aeruginosa* infections, as this drug has been shown to have potent inhibitory activity against the class C β-lactamase of *P. aeruginosa*, the *Pseudomonas*-derived cephalosporinase (PDC) (9). *P. aeruginosa* is a significant pathogen in hospital-acquired infections causing up to 10% of nosocomial infections and is notoriously difficult to treat with current antibiotic therapy (12–14). PDC expression is closely tied to cell wall recycling and can be induced after exposure to certain β-lactam antibiotics or hyperexpressed after various gene mutations or deletions (Fig. 1C) (15–20). Intrinsically, the membrane of *P. aeruginosa* has a 12- to 100-fold-lower permeability than *Escherichia coli*, making it difficult for antibiotics to pass through the bacterial outer membrane (18). In addition, *P. aeruginosa* possesses at least 60 porins, which mediate entry of antibiotics through the outer membrane and a loss of these porins affects antibiotic susceptibility of the bacteria, particularly the loss of OprD with respect to carbapenem susceptibility (17, 18, 20, 21). Therefore, the design of novel antibiotics that do not increase expression of the AmpC enzyme, are stable to hydrolysis by PDC, and are not susceptible to efflux is essential for combating MDR *P. aeruginosa* infections.

For the reasons enumerated above, we were compelled to explore the activity of β-lactam/avibactam combinations against a variety of *P. aeruginosa* clinical isolates and uncover any possible resistance determinants for β-lactam/avibactam combinations. We expanded on previous ceftazidime-avibactam testing against *P. aeruginosa* by performing a genetic, biochemical, and microbiological characterization (22–24). As part of our testing we selected: (i) an archived sample of strains collected >10 years before the release of ceftazidime-avibactam to determine whether there was any intrinsic resistance to this drug combination and (ii) genetically modified strains (transposon knockouts) that test a variety of components important to the β-lactam resistome of *P. aeruginosa*. In our study, nearly 20% of archived clinical isolates of *P. aeruginosa* obtained from a tertiary care center in northeast Ohio were found to be resistant to ceftazidime-avibactam combination (defined as an MIC > 8 mg/liter). Since selective pressure against avibactam was unlikely to play a role in resistance here, we hypothesized that our observed ceftazidime-avibactam resistance is due to a combination of drug efflux and decreased cell permeability. Our investigations revealed the importance of this finding in the introduction of novel agents that target *P. aeruginosa* and the implications this has for future therapy.

MATERIALS AND METHODS

Clinical isolates. A total of 54 isolates of *P. aeruginosa* were obtained from an archived library of *P. aeruginosa* clinical samples at the Cleveland Clinic Foundation collected between 2005 and 2008. These isolates were collected and archived due to a β-lactam-resistant phenotype. The isolates are listed in Table S1 in the supplemental material.

Genetic characterization of clinical isolates. Draft whole-genome sequencing was performed for ten ceftazidime-avibactam-resistant isolates (defined as an MIC of >8 mg/liter, which is the Clinical and Laboratory Standards Institute (CLSI) breakpoint for ceftazidime (since a ceftazidime-avibactam breakpoint has not yet been defined [25]) and is described in detail in the supplemental material.

MICs. Agar dilution MICs were performed according to the CLSI guidelines as previously described (25, 26). Briefly, Mueller-Hinton (MH) agar plates were poured with increasing concentrations of antibiotic and with avibactam held constant at 4 mg/liter in experiments where avibactam was used (27). A Steers replicator was used to deliver 10 μl of a culture containing 10^4 bacteria in MH broth. Plates were incubated overnight at 37°C and read the following day. The MIC was defined as the concentration of antibiotic where visible growth was absent. MICs were performed on the 54 clinical *P. aeruginosa* isolates, a control *Escherichia coli* DH10B strain containing the pseudomonal AmpC enzyme PDC-3 on a pBC SK(−) plasmid, and a control *P. aeruginosa* strain PAO1. We tested ampicillin (AMP), AMP-avibactam (AVI), ceftazidime (CAZ), CAZ-AVI, cefepime (FEP), aztreonam (ATM), piperacillin (PIP)-tazobactam (TZP), ciprofloxacin (CIP), gentamicin (GEN), imipenem (IPM), meropenem (MEM), colistin (COL), and fosfomycin (FOS). We also tested combinations of antibiotics with colistin at a constant 0.5 mg/liter, with CCCP (carbonyl cyanide *m*-chlorophenyl hydrazine) at a constant 12.5 μM and with PAβN (phenylalanine-arginine β-napthylamide) at a constant 50 mg/liter according to a previously published protocol used to evaluate *P. aeruginosa* (28). For experiments with fosfomycin, doubling dilutions of both ceftazidime and fosfomycin were performed in tandem and avibactam was held constant at 4 mg/liter, the reported MIC is the concentration at which cell growth was no longer observed for both fosfomycin and ceftazidime. The chemical structure of all tested compounds is shown in Fig. S4 in the supplemental material.

Transposon library. A transposon library containing PAO1-derived *P. aeruginosa* strains with transposon insertions in a variety of potential resistance genes was obtained from the University of Washington (29). A list of the bacterial strains tested is shown in Table S2 in the supplemental material. MICs were also performed on this library with ampicillin and ampicillin-avibactam as the strains were all already susceptible to ceftazidime.

AmpC expression levels. The expression levels of the AmpC β-lactamase enzymes were measured by performing a Western blot on the isolates. A complete description is provided in the supplemental material.

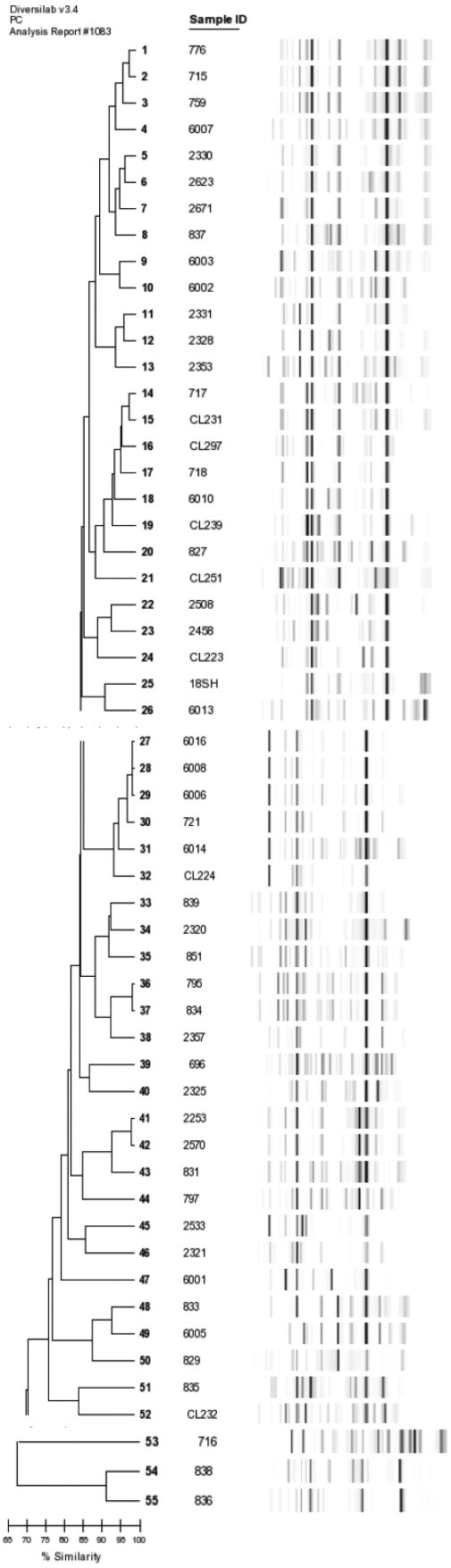
Enzyme purification and inhibitory kinetics measurements. The purification of PDC-1 and PDC-3 and inhibitory enzyme kinetics by avibactam are described in detail in the supplemental material.

Molecular modeling. The amino acid sequences of *P. aeruginosa* PBP1a (GenBank accession no. NP_253732.1) and *P. aeruginosa* PBP1b (GenBank accession no. NP_253388.1) were used to create models of these enzymes (see Fig. S2B and S3B in the supplemental material) using SwissModel software (http://swissmodel.expasy.org) (30–33).

RESULTS AND DISCUSSION

Genetic relatedness of *P. aeruginosa* clinical isolates. We studied an archived collection of *P. aeruginosa* as our goal was to assess whether strains with resistance to ceftazidime-avibactam were present before the drug combination is released in the clinic. Our collection of 54 clinical isolates was obtained from patients hospitalized between 2003 and 2008 (see Table S1 in the supplemental material) and was characterized to determine the genetic relatedness with repetitive extragenic palindromic (rep)-PCR. A dendrogram of the relationship between the *P. aeruginosa* strains is shown in Fig. 2. As is evident, the strains represent many heterogeneous clones. Multilocus sequence typing was also performed for several of the *P. aeruginosa* strains, and the results are included in Table S1 in the supplemental material. These results confirm that these clinical isolates represent different strains.

Activity of ceftazidime-avibactam combination chemotherapy against *P. aeruginosa* clinical isolates and identification of ten resistant strains. The isolates in Table S1 were initially tested against ampicillin, ampicillin-avibactam, ceftazidime, and ceftazidime-avibactam (Table 1). In general, there was a dramatic increase in susceptibility when avibactam was added to both ampicillin and ceftazidime. This is particularly significant for ampicillin as a decrease in MICs using a β-lactam antibiotic with minimal activity against *P. aeruginosa* is notable. Unexpectedly,



nine clinical isolates were identified in which avibactam was unable to significantly lower the MIC against ampicillin or ceftazidime and restore antibiotic susceptibility (for ceftazidime defined as an MIC of <8 mg/liter) (25). One additional strain was tested with an intermediate MIC of 8 mg/liter for ceftazidime-avibactam (strain 696). Mouse models and pharmacokinetic/pharmacodynamic studies of ceftazidime-avibactam indicate that an MIC above 8 mg/liter may result in clinical failure of this drug combination (23, 34, 35). As a result, these ten strains were chosen for further characterization.

Activity of β-lactam/avibactam combinations against a library of transposon knockouts. In order to help identify candidate genes that may lead to resistance to avibactam in clinical isolates, we next tested ampicillin-avibactam combinations against a library of transposon knockouts (see Table S2 in the supplemental material). These isolates possessed insertion elements in efflux pumps, porins, AmpC regulation elements, and penicillin-binding proteins (PBPs). The ampicillin MIC is lowered for all of the transposon knockouts after the addition of avibactam. We draw attention to important results regarding avibactam potency from this table. The OprD transposon mutant (PA0958) had a lower ampicillin-avibactam MIC (32 mg/liter), indicating that loss of the OprD porin is likely not a major resistance mechanism to avibactam in *P. aeruginosa*. In addition, as expected, hyperproduction of the PDC β-lactamase (in the AmpD transposon mutant, PA4522) reduces the susceptibility to ampicillin-avibactam (MIC of 512 mg/liter). In contrast, a potentiation of ampicillin activity was observed when avibactam was added to the AmpC transposon mutant (PA4110) with an MIC change from 128 mg/liter for ampicillin alone and 16 mg/liter after the addition of avibactam. The mechanism of this potentiation in the absence of a β-lactamase enzyme is not known. However, this phenomenon has previously been reported with a small synergistic effect of avibactam alone being observed in some bacterial isolates (36). In addition, when liquid cultures were grown with 40 mg of avibactam/liter alone, a noticeable drop in cell density was observed, indicating that avibactam likely has some antibacterial effect without a partner β-lactam against these *P. aeruginosa* strains. Overall, we did not observe a pattern where a transposon mutant of any of the efflux pumps affected susceptibility to ampicillin-avibactam. Therefore, our investigation of a defined collection of transposon mutants did not reveal potential β-lactam/avibactam resistance mechanisms among the targeted efflux pumps.

Exploring possible β-lactamase-mediated resistance mechanisms to ceftazidime-avibactam in the *P. aeruginosa* isolates. We next investigated whether differences in the primary amino acid sequence of the PDC β-lactamase impact susceptibility to ceftazidime-avibactam. To answer this, we sequenced the bla_{PDC} genes of each isolate (see Table S1 in the supplemental material). We found a variety of PDC enzymes present in our isolates with 13 different PDC enzymes represented. The ten isolates resistant to ceftazidime-avibactam had different AmpC enzymes and these same enzymes were also present in susceptible isolates, for example, PDC-1 in isolates PAO1 (susceptible) and 715 and 776 (resis-

FIG 2 Diversilab rep-PCR dendrogram analysis of the 54 clinical *P. aeruginosa* strains used in these experiments with the wild-type *P. aeruginosa* 18SH (which has a stably derepressed AmpC) included for comparison.

TABLE 1 MICs of various *E. coli* and *P. aeruginosa* clinical isolates[a]

Isolate	MIC (mg/liter)[b]											
	AMP	AMP-AVI	CAZ	CAZ-AVI	FEP	ATM	IPM	MEM	TZP	GEN	CIP	COL
E. coli												
DH10B	0.5	0.25	0.12	<0.06	<0.06	0.25	2	<0.06	<0.06	0.5	0.12	<0.06
DH10B bla_{PDC-3}	0.25	0.25	0.25	0.25	<0.06	0.25	2	<0.06	<0.06	0.5	0.12	<0.06
P. aeruginosa												
PAO1	4,096	32	2	1	1	1	2	0.5	1	2	1	0.5
2458	1,024	128	1	2	4	8	8	0.5	0.25	>128	8	0.25
2508	2,048	64	0.5	1	1	1	1	16	1	128	4	2
2533	2,048	128	1	1	2	2	2	0.5	1	4	32	2
2570	8,192	1,024	8	4	16	16	32	32	64	8	4	0.5
2623	8,192	128	16	2	8	2	16	32	1	>128	16	1
2671	8,192	512	16	2	16	16	64	64	1	4	16	1
6001	4,096	256	1	1	2	2	4	0.25	0.5	1	4	0.12
6002	8,192	128	2	2	2	2	2	0.5	0.5	4	1	0.25
6003	8,192	1,024	32	4	16	16	128	32	1	2	16	1
6005	2,048	32	2	0.5	2	2	4	0.5	0.25	2	1	0.5
6006	4,096	128	2	2	2	2	2	0.25	0.25	2	2	2
6007	2,048	256	2	2	4	4	4	0.5	0.25	2	2	0.25
6008	2,048	64	1	1	2	2	4	0.25	0.5	4	0.5	0.12
6010	8,192	128	8	2	16	4	32	0.5	0.25	16	1	2
6013	2,048	128	2	1	2	2	2	0.25	0.25	4	1	0.25
6014	2,048	16	0.5	0.25	1	1	1	0.25	0.25	2	2	0.5
6016	2,048	64	1	1	2	2	4	0.25	1	4	1	0.12
CL223	2,048	512	2	1	8	8	32	8	8	4	0.5	2
CL224	2,048	256	2	0.5	4	1	16	4	8	4	32	1
CL231	4,096	512	2	2	8	16	16	16	4	1	32	1
CL232	**16,384**	**4,096**	**>32**	**32**	**128**	**>128**	**64**	**64**	**>128**	**2**	**16**	**2**
CL239	1,024	64	1	0.5	2	4	16	2	2	1	0.25	1
CL251	2,048	512	4	4	16	32	16	8	16	1	2	1
CL297	8,192	512	>32	4	32	32	32	16	>128	4	0.12	2
696	**16,384**	**512**	**32**	**8**	**32**	**16**	**32**	**16**	**128**	**4**	**0.5**	**2**
715	**16,384**	**4,096**	**>32**	**32**	**64**	**64**	**32**	**32**	**>128**	**>128**	**32**	**2**
716	**4,096**	**256**	**>32**	**>32**	**32**	**128**	**32**	**64**	**16**	**4**	**64**	**2**
717	16,384	1,024	16	2	16	16	128	1	2	8	16	1
718	8,192	128	1	1	2	4	2	2	1	2	2	2
721	2,048	64	1	1	2	4	4	0.25	2	4	32	1
759	8,192	2,048	8	2	32	16	64	32	0.5	>128	16	2
776	**16,384**	**4,096**	**>32**	**>32**	**64**	**128**	**64**	**64**	**128**	**>128**	**64**	**2**
795	**>16,384**	**8,192**	**>32**	**>32**	**>128**	**128**	**32**	**32**	**>128**	**>128**	**128**	**2**
829	2,048	64	4	0.5	8	0.12	4	128	1	8	8	1
835	**4,096**	**256**	**>32**	**32**	**16**	**128**	**32**	**16**	**16**	**1**	**2**	**2**
836	1,024	256	8	1	16	16	32	>128	1	0.25	0.5	1
838	4,096	256	1	1	4	4	4	0.5	0.5	4	8	0.5
839	**8,192**	**1,024**	**>32**	**>32**	**64**	**128**	**4**	**16**	**128**	**1**	**>128**	**2**
2330	1,024	64	1	1	8	16	4	2	8	8	0.5	1
2353	8,192	128	16	1	8	16	2	2	64	2	0.25	1
2331	4,096	1,024	4	4	8	32	4	4	16	16	1	2
2253	4,096	256	32	2	32	32	16	16	128	>128	16	1
2320	2,048	64	1	1	4	4	0.5	0.5	4	2	0.12	1
2321	16,384	1,024	32	4	32	32	64	1	1	2	1	2
2325	2,048	128	1	0.25	2	2	4	1	4	2	0.12	1
2357	2,048	32	0.5	0.25	4	1	2	0.5	2	4	64	1
797	512	512	8	4	8	8	16	64	1	>128	4	1
827	2,048	128	1	1	4	8	4	0.25	0.12	4	4	0.12
831	4,096	128	1	1	8	2	2	1	2	4	0.5	1
833	1,024	128	2	2	8	4	4	16	0.25	>128	8	0.25
834	**2,048**	**256**	**>32**	**16**	**32**	**128**	**32**	**16**	**16**	**4**	**>128**	**2**
837	2,048	32	0.5	0.5	2	4	2	0.25	2	2	0.12	0.5
851	**8,192**	**256**	**>32**	**16**	**32**	**32**	**32**	**32**	**128**	**32**	**64**	**0.25**
2328	2,048	64	0.25	0.25	2	1	4	0.5	2	1	0.12	0.5

[a] AVI was maintained at a constant concentration of 4 mg/liter in the β-lactam/AVI combinations. All MICs were determined in triplicate. Isolates that did not demonstrate a significant decrease in MIC when AVI was added to CAZ are indicated in boldface.

[b] $MIC_{90\ AMP}$ = 16,384 mg/liter, $MIC_{90\ AMP\text{-}AVI}$ = 2,048 mg/liter, $MIC_{90\ CAZ}$ = >32 mg/liter, $MIC_{90\ CAZ\text{-}AVI}$ = 32 mg/liter, $MIC_{90\ FEP}$ = 64 mg/liter, $MIC_{90\ ATM}$ = 128 mg/liter, $MIC_{90\ IPM}$ = 64 mg/liter, $MIC_{90\ MEM}$ = 64 mg/liter, $MIC_{90\ TZP}$ = 128 mg/liter, $MIC_{90\ GEN}$ = >128 mg/liter, $MIC_{90\ CIP}$ = 64 mg/liter, $MIC_{90\ COL}$ = 2 mg/liter.

tant) and PDC-20 in isolates 2357 (susceptible) and 795 and 834 (resistant). Therefore, our analysis suggested that amino acid substitutions in the AmpC enzyme are unlikely to be the main mechanism conferring resistance to ceftazidime or ceftazidime-avibactam since there was no correlation between the PDC enzyme and the MIC. These results are consistent with previous investigations that analyzed the activity of ceftazidime-avibactam against *P. aeruginosa* isolates containing 57 different AmpC enzymes (37).

We also queried the strains for the presence of additional β-lactamase enzymes. Draft whole-genome sequencing of our 10 ceftazidime-avibactam resistant *P. aeruginosa* isolates revealed that additional β-lactamase genes were not present, with the exception of the PoxB β-lactamase present on the chromosome of all *P. aeruginosa* strains. Gene-specific PCR for common β-lactamases in U.S. medical centers in the other 44 *P. aeruginosa* isolates revealed that the ceftazidime-avibactam-susceptible isolates also likely did not have other β-lactamase enzymes. Therefore, we do not believe that the ceftazidime-avibactam resistance we observed

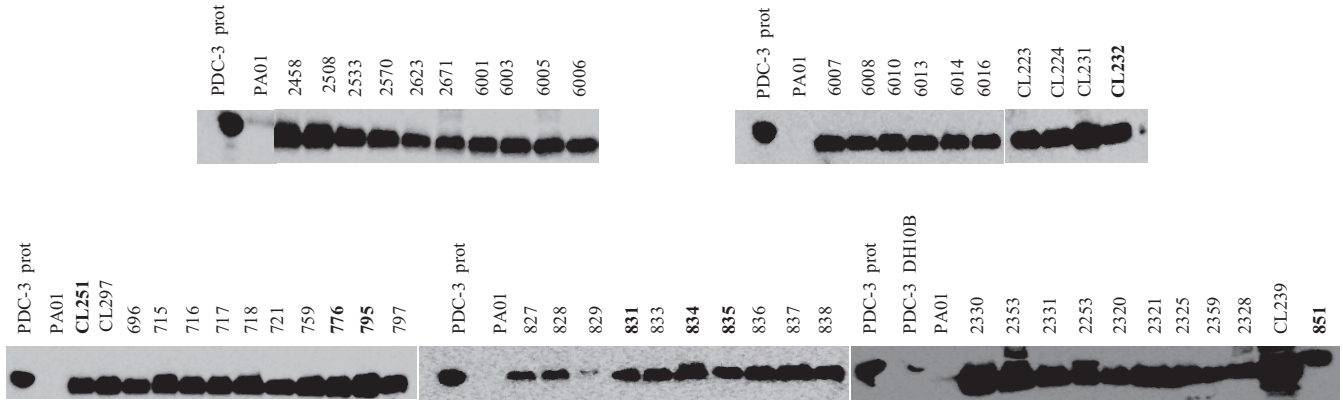


FIG 3 Western blot of the various *P. aeruginosa* clinical isolates tested with an antibody raised to the 18SH AmpC protein. Isolate names in boldface are strains resistant to CAZ-AVI combination treatment. "PDC-3 prot" is the purified PDC-3 protein as a size comparison for the AmpCs.

is due to the presence of additional β-lactamases in these isolates that are unable to be inhibited by avibactam such as the OXA class D enzymes or the class B metallo-β-lactamases.

Kinetic and biochemical characterization of different representative cephalosporinases. To confirm the above findings, since a large amount of variation in the primary amino acid sequence was observed in the AmpC enzymes of these clinical isolates, we pursued inhibitory kinetic comparison of two common representative variants of this enzyme, PDC-1 and PDC-3 by purifying both of these β-lactamases and assessing the ability of avibactam to inhibit each enzyme. The T105A substitution is the sole substitution in PDC-3 versus PDC-1; this substitution is also found in PDC-5, PDC-8, PDC-16/18, PDC-19, PDC-20, PDC-24, and PA14 and was previously reported to confer carbapenemase activity to the PDC β-lactamase (38). We found that the $K_{i\ app}$ (2.5 ± 0.3 versus 4.8 ± 0.5 μM), the k_2/K (29,000 M^{-1} s^{-1} versus 21,500 M^{-1} s^{-1}), and the k_{off} (0.0008 s^{-1} versus 0.0011 s^{-1}) were similar for PDC-3 and PDC-1, respectively (see Fig. S1 in the supplemental material). These measured kinetic constants support our above finding that the AmpC variation among *P. aeruginosa* may not be a significant barrier to avibactam activity and that avibactam will inhibit class C β-lactamases with "extended-spectrum" activity (37).

Comparative analysis of expression levels of AmpC, including the *bla*$_{AmpC}$ regulon. Since amino acid substitutions in the PDC β-lactamase do not appear to alter ceftazidime-avibactam MICs or avibactam inhibitory kinetics, we further examined the AmpC expression levels among the different clinical isolates (Fig. 3) (39). The PAO1 *P. aeruginosa* strain was used as a control, as its AmpC is not stably derepressed and therefore has a low expression level in the absence of a β-lactam inducer (40). We found that the AmpC expression level is relatively uniform among the *P. aeruginosa* strains. Overall, most of our clinical isolates exhibit increased AmpC enzyme production. One isolate (isolate 829) did not exhibit a stably derepressed AmpC.

As stated earlier, the AmpC enzyme is responsible for the expression of β-lactam resistance through a complex regulatory network (16, 39, 41, 42). Hyperexpression of this chromosomal class C β-lactamase involves the cell wall synthetic pathway and turnover, the AmpG permease, the AmpD amidase, and the AmpR transcriptional regulator (16, 17, 41, 42) (Fig. 1C). Increased production of the AmpC β-lactamase resulting in resistance to antipseudomonal penicillins and cephalosporins can result from exposure to certain β-lactams or BLIs, including imipenem, cefoxitin, and clavulanate, or from mutations in the *ampR* or *ampD* genes (41). Draft whole-genome sequencing revealed a variety of changes in the *ampR* and *ampD* genes when these strains were compared to PAO1 (see Table S3 in the supplemental material). One of these substitutions, a D135N or D135V substitution in *ampR* was previously shown to lead to increased production of AmpC in *Enterobacter cloacae* (43). However, the assessment of steady-state protein expression (i.e., the Western blot) does not seem to show a major difference in AmpC expression level in CL232 relative to the other strains, so this is probably not a significant resistance mechanism to ceftazidime-avibactam in this isolate.

Analysis of ceftazidime-avibactam-resistant *P. aeruginosa* clinical isolates: PBPs. In addition to the sequences of *ampR* and *ampD*, we examined the translated gene sequences of the cell wall synthesizing enzymes, PBPs, since these are the sites of action of β-lactams and substitutions in the targets of these antibiotics can lead to a lack of an effect of these drugs. We focused on four PBPs (PBP1a, PBP1b, PBP3a, and PBP4) in the ten isolates and compared them to the wild-type *P. aeruginosa* strain PAO1 PBP sequences.

When we compared the sequences of PBP1a and PBP1b among the ten isolates (see Table S3 in the supplemental material), none of the locations of amino acid substitutions were near the active-site residues, suggesting that these probably do not play a role in the observed β-lactam resistance pattern (see Table S3 and Fig. S2 and S3 in the supplemental material). Deletion of PBP3a has not been shown to affect *P. aeruginosa* antibiotic susceptibility, likely due to redundancy with PBP3 (44). Therefore, it seems unlikely that the few substitutions we observe in PBP3a (see Table S3 in the supplemental material) have an effect on the β-lactam resistance since the PBP3 genes are identical between all of the isolates and PAO1.

Inactivation of PBP4 has also been shown to lead to hyperproduction of AmpC in *P. aeruginosa*, leading to β-lactam resistance (45). Although we observe substitutions in the PBP4 amino acid sequence (see Table S3 in the supplemental material) these are probably not related to β-lactam/avibactam resistance since AmpC hyperproduction is present in both ceftazidime-avibactam-resistant and -susceptible isolates. Overall, the sequencing of the PBPs indicates that PBP protein production or function is not a major reason that these isolates show resistance to ceftazidime-avibactam.

TABLE 2 MICs of selected *E. coli* and *P. aeruginosa* clinical isolates with β-lactams supplemented with colistin, fosfomycin, PAβN, or CCCP

Isolate	MIC (mg/liter)[a]						
	CAZ-AVI-COL	FOS	FOS-CAZ-AVI	CAZ-PAβN	CAZ-AVI-PAβN	CAZ-CCCP	CAZ-AVI-CCCP
E. coli							
DH10B	–	32	–	<0.06	–	0.25	–
DH10B bla_{PDC-3}	–	32	<0.06	<0.06	<0.06	0.25	0.25
P. aeruginosa							
PAO1	0.5	64	1	0.5	<0.06	2	1
CL232	32	32	4	0.5	<0.06	256	64
696	8	128	1	4	0.25	64	8
715	32	8	8	32	2	256	64
716	4–8	128	64	128	128	4	2
776	2	128	4	128	128	256	16
795	128	128	4	8	0.25	256	64
834	8	64	4	1	0.25	4	4
835	2–4	64	4	1	0.25	128	64
839	8–16	>128	4	1	<0.06	64	8
851	–	>128	8	128	8	<0.06	<0.06

[a] Colistin was added to all plates at 0.5 mg/liter. Doubling dilutions of fosfomycin were performed in tandem with ceftazidime. PAβN was tested at 50 mg/liter. CCCP was tested at 12.5 μM. –, No growth.

Are permeability and efflux responsible for increased resistance? Since differences in the β-lactamase enzymes, β-lactamase expression level, or β-lactam targets (PBPs) did not seem to explain the ceftazidime-avibactam resistance among our isolates, we next focused our attention to membrane permeability and efflux as resistance mechanisms. Comparing the OprD porin amino acid sequences of our ten ceftazidime-avibactam-resistant isolates to the wild-type PAO1 strain, we found a variety of substitutions (see Table S3 in the supplemental material). Several of our isolates had OprD porin types corresponding to previously characterized substitutions which are listed in the table (46). In four isolates, frameshift substitutions in the gene sequence were observed and one did not properly align with the PAO1 sequence. As a result, the OprD porin is likely not expressed in these five isolates (CL232, 696, 715, 775, and 851) due to production of an incomplete protein. In the other five strains, there are a variety of amino acid substitutions and/or amino acid deletions that may also prevent proper protein folding and expression of the porin. A lack of OprD production was shown to lead to carbapenem resistance, particularly to imipenem, but to not have a large effect on the susceptibility to other antibiotics (48). Only isolate 839 shows a low MIC for imipenem (4 mg/liter, Table 1), indicating that OprD is probably only functional in this isolate.

Although our draft whole-genome sequencing shows the OprD porin is likely nonfunctional in most of our ceftazidime-avibactam-resistant isolates, this is probably not the major mechanism of resistance as our transposon mutant library indicates that OprD is noncontributory to avibactam susceptibility. To confirm this, we also sequenced the gene for *oprD* in our eight ceftazidime-avibactam-susceptible, carbapenem-resistant isolates (717, 2253, 2321, 2353, 2623, 2671, 6003, and CL297; see Table S4 in the supplemental material). We found several misaligned sequences among these isolates as well. This indicates that loss of the OprD porin is likely not the major mechanism of resistance to ceftazidime-avibactam as isolates which appear to have a nonfunctional porin have a low MIC to ceftazidime combined with avibactam.

In order to probe additional resistance mechanisms, we next analyzed the potency of different antibiotics and antibiotic combinations, including colistin, fosfomycin, and efflux pump inhibitors against these clinical isolates (Tables 1 and 2). We found that the isolates that showed ceftazidime-avibactam resistance also displayed resistance to all of the β-lactam antibiotics, and many of the non-β-lactam antibiotics (with the exception of gentamicin having a lower MIC for many of the strains, ≤4 mg/liter for six strains, and isolate 696 which is susceptible to ciprofloxacin). Notably, all of the tested *P. aeruginosa* clinical isolates were susceptible to colistin with MICs of ≤4 mg/liter. We therefore concluded that these *P. aeruginosa* strains likely have a broad-spectrum resistance mechanism due mainly to increased efflux pump production with a minor contribution from decreased porin expression.

The efflux pump system of *P. aeruginosa* is complicated with at least 12 different tripartite resistance-nodulation-division (RND) efflux pumps consisting of an outer membrane protein in combination with a periplasmic membrane fusion protein and a cytoplasmic membrane transporter (41, 49). The efflux pumps include MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM, MexJK-OprM, MexHI-OpmD, MexPQ-OpmE, MexMN-OprM, and MexVW-OprM (41, 50). *P. aeruginosa* also has ATP-binding cassette (ABC)-family pumps, small multidrug resistance family pumps, major facilitator superfamily pumps, and multidrug and toxic compound extrusion family pumps capable of removing toxic compounds and antibiotics from the bacteria (41). Upregulated MexAB-OprM and/or MexXY-OprA pumps often lead to resistance to meropenem (51). In addition, the upregulation of the MexXY efflux pump can also contribute to multidrug resistance among bacterial strains (including resistance to aminoglycosides, fluoroquinolones, and cefepime) (52). Recently, a report characterizing ceftazidime-resistant clinical isolates showed that the major mechanisms of resistance include decreased OprD production and increased MexAB-OprM expression in 32.5% of isolates, increased MexCD-OprJ expression in 8.3% of isolates, and increased MexXY-OprM expression in 28.4% of isolates (53). This leads us to believe that the MDR observed among our isolates is likely multifactorial due to the upregulation of multiple efflux pumps since a majority of the isolates were not susceptible to any single agent chemotherapy.

We further tested the contribution of efflux pump upregula-

tion in our strains by performing quantitative reverse transcription-PCR using primers for *mexA*, *mexE*, *mexX*, and *rpsL* as a housekeeping gene for expression comparison according to validated primer sequences (53). Unfortunately, we did not observe an upregulation of these pumps in our ten ceftazidime-avibactam-resistant strains compared to PAO1 or strain 717 which is carbapenem resistant but ceftazidime-avibactam susceptible with the exception of strain 851, which had upregulation of all three pumps. In addition, no changes in pump expression levels were observed after the addition of 4 mg/liter of avibactam to the growth medium. Therefore, the exact mechanism of resistance in these strains still remains to be elucidated.

To further assess the involvement of efflux pumps, we used efflux pump inhibitors, carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) and phenylalanine-arginine β-napthylamide (PAβN). CCCP is a nonspecific energy decoupler, which dissipates the proton motive force and destroys the energy gradient used for efflux by the RND-type pumps (Table 2) (54). PAβN (also known as MC-207,110) acts to specifically inhibit the Mex efflux pumps of *P. aeruginosa* without destroying the proton gradient of the cell and may also have some activity in permeabilizing the cell membrane (55).

CCCP lowered the ceftazidime-avibactam MICs for four isolates (716, 776, 834, and 839), PAβN lowered the ceftazidime-avibactam MICs for 7 isolates (CL232, 696, 715, 795, 834, 835, and 839).

As we have shown here, combination therapy with multiple antibiotic classes to overcome the resistance phenotype is essential to treating *P. aeruginosa* infections as previously proposed (15, 56). Likely, a cell wall active agent/BLI combination will need to be combined with an as-yet-identified agent to either increase permeability or prevent efflux in order to successfully eradicate MDR *P. aeruginosa* infections.

Exploring synergistic drug combinations against MDR *P. aeruginosa*. Colistin was previously shown to enhance the activity of antibiotic therapy against "hard-to-treat" MDR clinical isolates by combining colistin with another antibiotic (57). We tested colistin at a sub-MIC value of 0.5 mg/liter with ceftazidime or ceftazidime-avibactam against our resistant isolates to determine whether colistin would lead to synergy or an additive effect with these antibiotics and restored susceptibility to ceftazidime-avibactam in the resistant *P. aeruginosa* strains (Table 2). We found that seven of the ten strains had an MIC lowered to ≤16 mg/liter for ceftazidime-avibactam after colistin was added at 0.5 mg/liter. Since colistin has a bactericidal effect on its own, we also tested polymyxin B nonapeptide (PMBN) in combination with ceftazidime-avibactam in order to increase permeability without an independent bactericidal effect. We found that the ceftazidime-avibactam MICs were not lowered in combination with PMBN, which supports efflux as a greater mechanism of resistance versus porin loss among these isolates.

To further explore the multidrug resistance of and possibility of finding a potent drug combination for these ten *P. aeruginosa* strains, we tested fosfomycin as a synergistic drug in combination with ceftazidime-avibactam. Fosfomycin is a phosphoenol pyruvate mimic that interferes with bacterial cell wall synthesis before the step of β-lactam action (58, 59). Fosfomycin has been found to be an effective treatment for MDR *P. aeruginosa*, to exhibit synergistic effects with some antibiotics, and to increase the uptake of other antibiotics when it is given in combination with them (60–63). When the concentration of fosfomycin was increased in tandem with ceftazidime (and avibactam held constant), the MICs of all but three of the strains were lowered to below the breakpoint of <8 mg/liter, with two strains having an MIC of 8 mg/liter. Notably, the MICs of fosfomycin alone were still above the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint of this drug (≤32 mg/liter [58]) for most of the strains. Fosfomycin seems to be an optimal lead compound for combination chemotherapy with ceftazidime-avibactam to treat MDR *P. aeruginosa* since it lowered the MICs for 9/10 highly resistant *P. aeruginosa* strains to at or below the breakpoint for this drug combination.

Conclusion. Avibactam is a novel non-β-lactam BLI that inactivates class A, C, and some class D β-lactamases. Ceftazidime-avibactam is being proposed as a therapeutic agent against bacteria containing these enzymes in clinical trials. Here, we investigated the activity of this inhibitor in combination with ceftazidime against a variety of β-lactam-resistant *P. aeruginosa* clinical isolates from up to a decade ago and found that 18.5% of the archived isolates were also resistant to ceftazidime-avibactam with an MIC of ≥16 mg/liter. This is in contrast to previous studies using very large collections of *P. aeruginosa* isolates that were not selected on the basis of drug resistance, in which >90% of the *P. aeruginosa* isolates were susceptible to ceftazidime-avibactam (36, 64–66).

Although many *P. aeruginosa* isolates will be susceptible to ceftazidime-avibactam, clinical failure may occur when MDR isolates are identified as acquired resistance seems to be present to ceftazidime-avibactam in *P. aeruginosa*, which may be driven by altered outer membrane permeability or overexpressed efflux pumps. Adding colistin to the ceftazidime-avibactam combination poses a potential advantage to the treatment of MDR *P. aeruginosa* as resistance is reduced to only 7% of strains once colistin is added. Fosfomycin further improves the observations with colistin as resistance is only observed in 1.9% of strains when ceftazidime-avibactam-fosfomycin is tested. Novel drugs that exploit different mechanisms of action are needed for the treatment *P. aeruginosa*. Agents in development that may also prove useful for MDR bacteria include Lpx-C inhibitors (47, 67).

Bacterial infections due to MDR organisms are a significant threat to our antibiotic armamentarium requiring increasing imagination by clinicians for treatment. Here, we have defined the "resistome" of ten MDR *P. aeruginosa* isolates obtained by a clinical microbiology lab nearly a decade ago. We determined through a series of antibiotics that the major contribution of resistance is cell impermeability and efflux and not changes in β-lactamase sequence or expression level or changes in PBP sequence. By testing various antimicrobial agents, we propose a novel strategy for treatment of dangerous MDR infections using a triple chemotherapy involving β-lactam/BLIs combined with a third agent targeting impermeability such as an efflux pump inhibitor or a cell wall-active agent with a non-PBP target.

ACKNOWLEDGMENTS

We thank AstraZeneca Pharmaceuticals for providing the avibactam powder and for their support of the whole-genome sequencing and analysis.

M.L.W. was supported by a medical scientist training program training grant from Case Western Reserve University-T32 GM07250. K.P.W. was supported in part by funds and/or facilities provided by the Cleveland Department of Veterans Affairs and a Veterans Affairs Career Develop-

ment Award. R.A.B. was supported in part by funds and/or facilities provided by the Cleveland Department of Veterans Affairs, the Department of Veterans Affairs Merit Review Program 1I01BX001974, the Veterans Integrated Service Network 10 Geriatric Research, Education, and Clinical Center (VISN 10 GRECC), and the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under awards R01 AI100560 and R01 AI063517. Financial support for this study was provided by AstraZeneca Pharmaceuticals, Waltham, MA. R.A.B. also receives funding from Merck Pharmaceuticals, Cubist Pharmaceuticals, and Steris. R.A.B. is also a member of the Tetraphase data safety monitoring board.

All authors have reviewed the manuscript and approved it for submission.

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