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PHARMACOLOGY

Antibacterial Activity of Human Simulated Epithelial Lining Fluid Concentrations of Ceftazidime-Avibactam Alone or in Combination with Amikacin Inhale (BAY41-6551) against Carbapenem-Resistant *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*

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ABSTRACT The role of inhalational combination therapy when treating carbapenem-resistant *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* with newer beta-lactam/beta-lactamase inhibitors has not been established. Using a 72-h *in vitro* pharmacodynamic chemostat model, we simulated the human exposures achieved in epithelial lining fluid (ELF) following intravenous treatment with ceftazidime-avibactam (CZA) 2.5 g every 8 h (q8h) alone and in combination with inhaled amikacin (AMK-I) 400 mg q12h, a reformulated aminoglycoside designed for inhalational administration, against three *P. aeruginosa* isolates (CZA [ceftazidime/avibactam] MICs, 4/4 to 8/4 μg/ml; AMK-I MICs, 8 to 64 μg/ml) and three *K. pneumoniae* isolates (CZA MICs, 1/4 to 8/4 μg/ml; AMK-I MICs, 32 to 64 μg/ml). Combination therapy resulted in a significant reduction in 72-h CFU compared with that of CZA monotherapy against two of three *P. aeruginosa* isolates (-4.14 $\log_{10}$ CFU/ml, $P = 0.027$; -1.42 $\log_{10}$ CFU/ml, $P = 0.020$; and -0.4 $\log_{10}$ CFU/ml, $P = 0.298$) and two of three *K. pneumoniae* isolates (0.04 $\log_{10}$ CFU/ml, $P = 0.963$; -4.34 $\log_{10}$ CFU/ml, $P < 0.001$; and -2.34 $\log_{10}$ CFU/ml, $P = 0.021$). When measured by the area under the bacterial growth curve (AUBC) over 72 h, significant reductions were observed in favor of the combination regimen against all six isolates tested. AMK-I combination therapy successfully suppressed CZA resistance development in one *K. pneumoniae* isolate harboring bla_{KPC-3} that was observed during CZA monotherapy. These studies suggest a beneficial role for combination therapy with intravenous CZA and inhaled AMK when treating pneumonia caused by carbapenem-resistant Gram-negative bacteria.

KEYWORDS aminoglycoside, cephalosporin, pharmacodynamics

Hospital-acquired and ventilator-associated pneumonia (HAP and VAP, respectively) remain significant causes of morbidity and mortality in critically ill patients (1, 2). Intubation and mechanical ventilation are often required in patients with critical illness, and 9 to 27% of these intubated patients will develop VAP (3). Mortality attributable to VAP varies substantially in the literature but may be as high as 50 to 69% in some reports (4, 5). To date, Gram-negative bacteria account for the majority of isolated pathogens causing HAP and VAP (2), with *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* being the most common Gram-negative pathogens (6). These Gram-negative rods are among those bacteria included in the Infectious Diseases Society of America's (IDSA's) ESKAPE acronym defining isolates of medical concern that are frequently multidrug resistant (MDR) (7). In recent U.S. surveillance of isolates collected

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from patients with HAP and VAP, 21% of *P. aeruginosa* isolates were resistant to carbapenems; furthermore, 18% met the definition of MDR, being resistant to at least one agent in three or more antibiotic classes. Similarly, carbapenem resistance in *Klebsiella* spp. was 6.6% (6). Due to a lack of antibiotics retaining *in vitro* activity, successful treatment of HAP and VAP due to MDR *P. aeruginosa* and *K. pneumoniae* has become a concerning challenge.

Ceftazidime-avibactam (CZA) was the first beta-lactam/beta-lactamase inhibitor combination approved with activity against *Enterobacteriaceae* possessing the *Klebsiella pneumoniae* carbapenemase (KPC) beta-lactamase (8). Avibactam also restores ceftazidime activity in ~80% of ceftazidime-nonsusceptible *P. aeruginosa* isolates and was active against 86.3% of meropenem-nonsusceptible isolates (6). Since its approval for the treatment of complicated urinary tract and intra-abdominal infections, ceftazidime-avibactam has demonstrated noninferiority to meropenem in the treatment of nosocomial pneumonia, including VAP (9). Despite these positive results, the overall in-hospital mortality in 60 patients with carbapenem-resistant *Enterobacteriaceae* (CRE) infections in one report was 32%, and mortality was highest for patients with pneumonia (10). No difference was observed between patients receiving monotherapy or combination therapy. A separate report in 109 patients with carbapenem-resistant *K. pneumoniae* bacteremia observed significantly higher clinical success and survival than that with standard of care (typically carbapenems plus colistin or aminoglycosides) (11). Notably, among the 13 patients treated with ceftazidime-avibactam, success rates were 75% and 100% among the 8 and 5 patients who received monotherapy and combination therapy, respectively. Sparse data are available in the treatment of severe *P. aeruginosa* infections. A role for combination therapy against these MDR organisms may be justified, given the severity of infection and lack of alternative antibiotics. Indeed, a statement from several European societies on the management of KPC-producing *K. pneumoniae* infections recommends combination therapy in cases of severe infections (12).

When selecting a combination therapy regimen, penetration to the site of infection is paramount to successful treatment of HAP and VAP. Both ceftazidime and avibactam demonstrated penetration into the epithelial lining fluid (ELF) of healthy volunteers that was ~31 to 35% free plasma (13). Combination therapy with an aminoglycoside, as done in many studies, is challenged by systemic toxicity on top of reduced penetration into ELF (14). Amikacin Inhale (BAY41-6551) is a special formulation of amikacin for inhalation delivery through a proprietary vibrating-mesh nebulizer, the pulmonary drug delivery system (PDDS) (15, 16). The PDDS nebulizer can be integrated with standard mechanical ventilators to deliver 50 to 70% of base amikacin into the lower airways (17). *In vitro* pharmacodynamic studies have also observed antibacterial activity of amikacin alone at these exposures up to MICs of 256 μg/ml (18). Amikacin Inhale is currently in phase 3 development as adjunctive therapy to standard intravenous antibiotics for the treatment of pneumonia in patients who are mechanically ventilated.

The current study aimed to evaluate the antibacterial activity of observed human simulated ELF exposures of intravenous ceftazidime-avibactam alone or in combination with inhaled amikacin against MDR *P. aeruginosa* and *K. pneumoniae*.

RESULTS

Isolates. Ceftazidime-avibactam and amikacin MICs for selected isolates are provided in Table 1. All isolates were susceptible to ceftazidime-avibactam, and all had MICs of 4/4 to 8/4 μg/ml, with the exception of one *K. pneumoniae* isolate, which had an MIC of 1/4 μg/ml. All *K. pneumoniae* isolates possessed the bla_{KPC} gene, with one producing KPC-2 and two producing KPC-3 enzymes. Two of the six isolates were amikacin susceptible, while the remaining four displayed MICs of 32 to 64 μg/ml. All amikacin-nonsusceptible isolates possessed genes encoding aminoglycoside-modifying enzymes.

Antibiotic exposures. Observed free ceftazidime and amikacin concentrations were similar to predicted values (Fig. 1a and b). As noted in Materials and Methods, avibactam concentrations were not measured but were calculated as one-fourth that of

TABLE 1 Genotypes and MICs for amikacin and ceftazidime-avibactam for *P. aeruginosa* and *K. pneumoniae* isolates selected for the study

Isolate	Genotype	Amikacin MIC (μg/ml)	Ceftazidime-avibactam MIC (μg/ml)[a]
P. aeruginosa			
C42-72	Unknown	8	8/4
C42-45	Unknown	16	8/4
1504	*aph(3′)-IIb* bla_{OXA-50} *catB4 fosA1*; probable MexXY-OprM mutation(s)	64	4/4
K. pneumoniae			
329b	*ant(3′)-Ia ant(2′)-Ia aadA1 aadA2 aac(6′)-Ib aph(3′)-Ia aph(4′)-Ia fosA9 catA1* bla_{SHV-11} bla_{SHV-5} bla_{OXA-9} bla_{TEM-1} bla_{KPC-2} *mphA dfrA12 sul1 sul3*	32	1/4
375	*ant(3′)-Ia ant(2′)-Ia aac(6′)-Ib* (two copies) *strA strB aadA1 aph(3′)-Ia fosA9 catA1* bla_{SHV-5} bla_{OXA-9} bla_{TEM-1A} bla_{KPC-3} *mphA dfrA12 dfrA14 sul1 sul2*	64	4/4
352	*aac(6′)-Ib C aph(3′)-Ia fosA9 catA1* bla_{SHV-11} bla_{KPC-3} *dfrA12 sul1*	64	8/4

[a]Ceftazidime-avibactam MICs were determined in the presence of a fixed concentration of avibactam at 4 μg/ml.

ceftazidime. Targeted and observed (calculated for avibactam) pharmacodynamic exposures for amikacin, ceftazidime, and avibactam are presented in Table 2.

Antibacterial activity. The mean bacterial density of the starting inoculum for all experiments was 6.57 $\pm$ 0.28 $\log_{10}$ CFU/ml. Control isolates grew to 7.08 $\pm$ 0.47 and 8.39 $\pm$ 0.18 $\log_{10}$ CFU/ml by 72 h, respectively. Figure 2a to c portrays the comparative bacterial growth curves over the duration of the experiment for the *P. aeruginosa* isolates after exposure to no antibiotic (control), ceftazidime-avibactam alone, and ceftazidime-avibactam plus amikacin. Figure 3a to c portrays the comparative bacterial growth curves for the *K. pneumoniae* isolates.

Table 3 contains 72-h CFU results for each isolate. Against *P. aeruginosa*, ceftazidime-avibactam alone reduced CFU compared with that of the control for *P. aeruginosa* C42-45 ($P = 0.026$) and 1504 ($P = 0.006$), although organisms started to regrow by 72 h (Fig. 2b and c); activity against C42-72 was similar to that of the control ($P = 0.472$). The combination of ceftazidime-avibactam plus amikacin significantly reduced 72-h CFU values in two of the three isolates (*P. aeruginosa* C42-72, -4.14 $\log_{10}$ CFU/ml, $P = 0.027$; *P. aeruginosa* C42-45, -1.42 $\log_{10}$ CFU/ml, $P = 0.020$). The observed mean change in $\log_{10}$ CFU/ml for *P. aeruginosa* 1504 was -0.4 ($P = 0.298$) between combination and monotherapy.

Against *K. pneumoniae* isolates, ceftazidime-avibactam alone reduced CFU values compared with that of the control against all isolates ($P < 0.05$); however, similar to *P. aeruginosa*, isolate regrowth was observed (Fig. 3a to c). The combination of ceftazidime-avibactam plus amikacin significantly reduced 72-h CFU values in two of three isolates (*K. pneumoniae* 375, -4.34 $\log_{10}$ CFU/ml, $P < 0.001$; *K. pneumoniae* 352, -2.34 $\log_{10}$ CFU/ml, $P = 0.021$). Against *K. pneumoniae* 329b, combination therapy resulted in an 0.04 $\log_{10}$ CFU/ml increase ($P = 0.963$).

Area under the bacterial growth curves (AUBC) over 72 h for each isolate are provided in Table 4. For all tested isolates, the ceftazidime-avibactam plus amikacin combination regimen significantly reduced the AUBC compared with that of ceftazidime-avibactam alone. When evaluating the results shown in Fig. 2 and 3, much of this enhanced antibacterial effect with the amikacin combination can be attributed to a more rapid decline in CFU over the first few hours of each experiment than with ceftazidime-avibactam alone.

Resistance emergence. There were no changes in ceftazidime-avibactam MICs following the monotherapy experiments against the tested isolates, with the exception of *K. pneumoniae* 352. *K. pneumoniae* 352 had a baseline ceftazidime-avibactam MIC of 8/4 μg/ml and was found to express bla_{KPC-3} and bla_{SHV-11}. The ceftazidime-avibactam MIC increased to 64/4 μg/ml after 24 h of exposure to monotherapy and remained at this level throughout 48- and 72-h MIC assessments. The addition of amikacin to ceftazidime-avibactam against *K. pneumoniae* 352 prevented an increase in MIC throughout the experiment. No changes in amikacin MIC were observed for any isolates during the combination regimen studies.

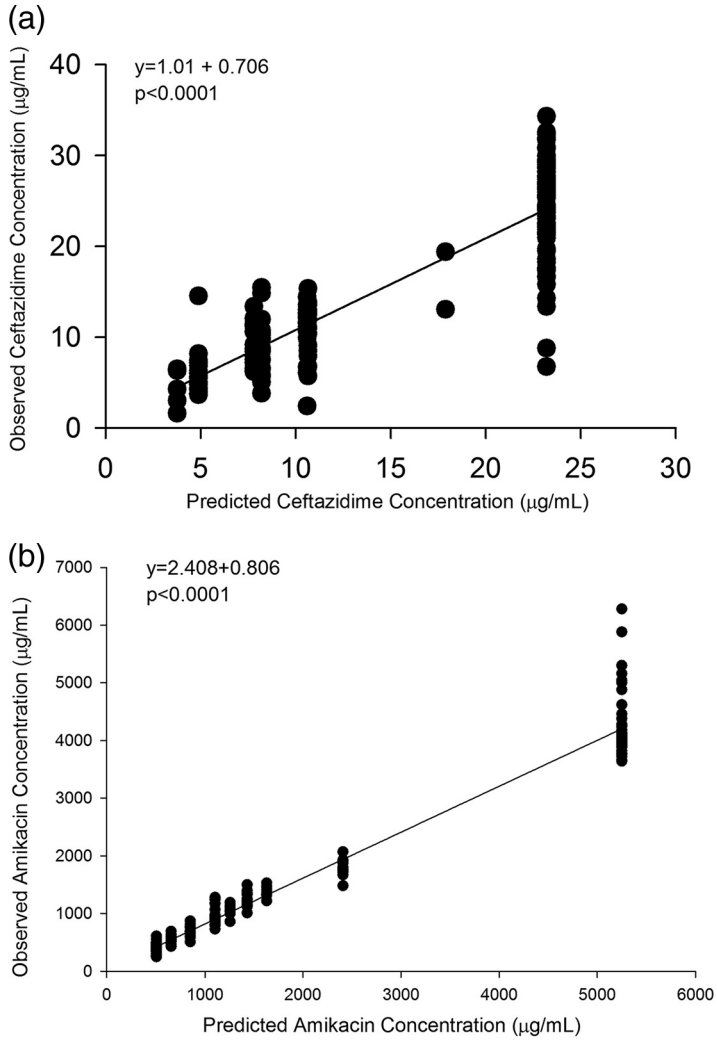

FIG 1 Observed versus predicted ceftazidime (a) and amikacin (b) concentrations in the *in vitro* pharmacodynamic model.

DISCUSSION

HAP and VAP caused by MDR Gram-negative bacteria remain challenging infections to treat effectively. Ceftazidime-avibactam has activity against many isolates of carbapenem-resistant *P. aeruginosa* and *K. pneumoniae*, the two most common Gram-negative bacteria implicated in HAP and VAP (6). Given the few antibiotic alternatives available and the challenges in achieving therapeutic concentrations at the site of pulmonary infections, exploring the role of combination therapy is justified, particularly with agents administered via inhalation, as recommended by the most recent IDSA guidelines (1). Amikacin Inhale combines a reformulated amikacin delivered via a proprietary nebulizer to achieve enhanced exposure in the lower airways (15–17). We used the *in vitro* pharmacodynamic chemostat model to determine the added antibacterial activity of combination therapy with ceftazidime-avibactam plus amikacin in comparison with the activity of ceftazidime-avibactam alone against carbapenem-resistant *P. aeruginosa* and *K. pneumoniae*. The combination regimen increased activity in four of six isolates over that of monotherapy, including the suppression of the emergence of ceftazidime-avibactam resistance in one *K. pneumoniae* strain.

Six carbapenem-resistant MDR Gram-negative isolates were selected for this experiment, all having ceftazidime-avibactam MICs in the susceptible, yet clinically relevant, range. The three *P. aeruginosa* isolates in this study all had ceftazidime-avibactam MICs

TABLE 2 Target versus observed pharmacodynamic parameters achieved in the *in vitro* pharmacodynamic model[a]

Isolate	Amikacin		Ceftazidime		Avibactam	
	Target *f*AUC/MIC	Observed *f*AUC/MIC	Target $fT_{>MIC}$ (%)	Observed $fT_{>MIC}$ (%)	Target $fT_{>1\ \mu g/ml}$ (%)[c]	Calculated $fT_{>1\ \mu g/ml}$ (%)[d]
P. aeruginosa						
C42-72	4,482	3,379 ± 404	90.6	76.2 ± 22.3[b]	75.8	93.9 ± 8.6
C42-45	2,241	1,909 ± 111	90.6	96.7 ± 0.5	75.8	99.6 ± 0.8
1504	560	425 ± 17	100	99.4 ± 0.8	75.8	99.5 ± 0.7
K. pneumoniae						
329b	1,120	881 ± 10	100	100.0	75.8	98.8 ± 2.2
375	560	492 ± 3	100	99.7 ± 0.3	75.8	99.8 ± 0.3
352	560	465 ± 10	90.6	96.3 ± 3.8	75.8	100.0

[a]Observed pharmacodynamic exposure reported as mean ± standard deviation of results from two to four observations.
[b]One ceftazidime-avibactam experiment (when studied in combination with amikacin) resulted in less than the target ceftazidime $fT_{>MIC}$ due to low concentrations in one 8-h interval. The bacterial time-kill curve did not appear different from its duplicate run, and exposure was still above 50% $fT_{>MIC}$ and was observed only in the combination run, so this experiment was included.
[c]Target $fT_{>1\ \mu g/ml}$ values for avibactam were derived from the ELF concentration profile (13).
[d]Avibactam concentrations were not measured in the experiment but were calculated as one-fourth of ceftazidime concentrations due to the use of a formulation with a 4:1 ceftazidime/avibactam concentration ratio. Note that the pharmacodynamic target exposure for avibactam is at least 50% $fT_{>1\ \mu g/ml}$, and therefore this exposure was achieved in all experiments.

of 4/4 to 8/4 μg/ml, which is within 1 dilution of the susceptibility breakpoint. *P. aeruginosa* isolates with these MICs are not uncommon. In a recent U.S. surveillance study of respiratory isolates, 14.8% and 6.1% of all 3,402 *P. aeruginosa* isolates had ceftazidime-avibactam MICs of 4/4 and 8/4 μg/ml, respectively (6). These frequencies were 31.1% and 19.3% for meropenem-nonsusceptible *P. aeruginosa* isolates. The modal ceftazidime-avibactam MIC for carbapenem-resistant *Klebsiella* species was 1/4 μg/ml (30% of the population) and thus is represented by *K. pneumoniae* 329b in our study. However, approximately 1% of isolates harbored MICs of 4/4 to 8/4 μg/ml during surveillance (6). These *Klebsiella* isolates with susceptible but higher ceftazidime-avibactam MICs are more likely to possess mutations in bla_{KPC-3} and have been associated with clinical failure and emergence of resistance (19, 20).

The amikacin MICs for these selected isolates spanned a broad range of 8 to 64 μg/ml. In the same surveillance study (6), the amikacin MIC_{90}s for *P. aeruginosa* and all *Enterobacteriaceae* were 16 and 4 μg/ml, respectively. Very few Gram-negative isolates (<5%) display amikacin MICs of 32 to 64 μg/ml (21); furthermore, exposures obtained with Amikacin Inhale have been shown to display antibacterial activity against *P. aeruginosa* and *K. pneumoniae* with MICs of up to 256 μg/ml (18). Many genes encoding aminoglycoside-modifying enzymes were observed in our isolates, including *aac(6′)-Ib* and *aph(3′)-Via*, and these were likely responsible for the increased amikacin MICs of some isolates.

Given the susceptibility of all *P. aeruginosa* isolates, we expected to observe significant early CFU reductions over the first 12 to 24 h following ceftazidime-avibactam monotherapy. This was followed, however, by slow regrowth by 72 h, particularly against *P. aeruginosa* C42-72 and C42-45, both of which had MICs of 8/4 μg/ml and grew back nearly to control values (Fig. 2a and b). This was despite very high percentage of dosing interval in which free drug concentration remained above the MIC ($fT_{>MIC}$) (ceftazidime, >90% $fT_{>MIC}$, and avibactam, >90% $fT_{>1\ \mu g/ml}$) in all monotherapy experiments. These observations against *P. aeruginosa* with MICs of 8/4 μg/ml are similar to those of previous hollow-fiber experiments conducted over 24 h, where initial kill was followed by regrowth to control levels (22, 23). In contrast, isolates with MICs of 4/4 μg/ml typically result in multiple logs of kill after exposure to 2.5 g every 8 h as a 2-h infusion (22, 23), which was the case with *P. aeruginosa* 1504. The addition of amikacin resulted in a quicker kill to the lower level of detection in two of the three *P. aeruginosa* isolates (Fig. 2a and c) and significantly lower ($P = 0.027$ and $P = 0.020$) 72-h CFU values than with monotherapy for both isolates with MICs of 8/4 μg/ml. As noted, the single isolate (*P. aeruginosa* 1504) that did not achieve a signifi-

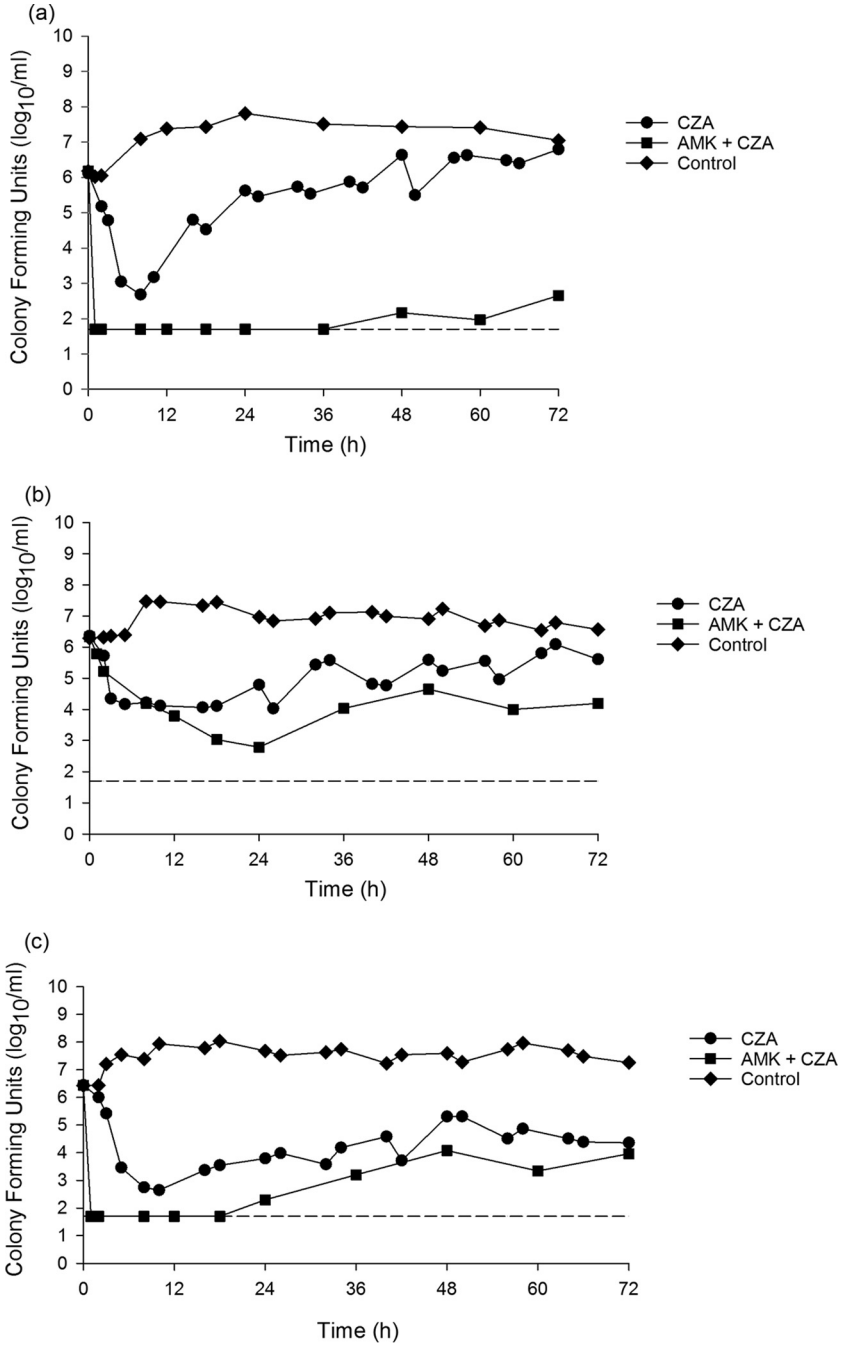

FIG 2 Mean bacterial densities over 72 h for ceftazidime-avibactam (CZA) alone and CZA plus amikacin (AMK) combination therapy against *P. aeruginosa* C42-72 (CZA MIC, 8 μg/ml; AMK MIC, 8 μg/ml) (a), *P. aeruginosa* C42-45 (CZA MIC, 8 μg/ml; AMK MIC, 16 μg/ml) (b), and *P. aeruginosa* 1504 (CZA MIC, 4 μg/ml; AMK MIC, 64 μg/ml) (c). Dashed line, lower limit of detection.

cant reduction after exposure to the combination had already achieved a substantial decline in CFU with ceftazidime-avibactam monotherapy; moreover, the amikacin MIC was 64 μg/ml, resulting in an observed amikacin free drug area under the curve-to-MIC ratio (*f*AUC/MIC) of 425 and which may not have been sufficient to result in a significantly greater kill against this isolate.

Against the *K. pneumoniae* isolates, ceftazidime-avibactam monotherapy again resulted in early CFU reductions for the first 12 to 24 h, followed by regrowth in all isolates, and in one case the development of ceftazidime-avibactam resistance. In contrast to observations

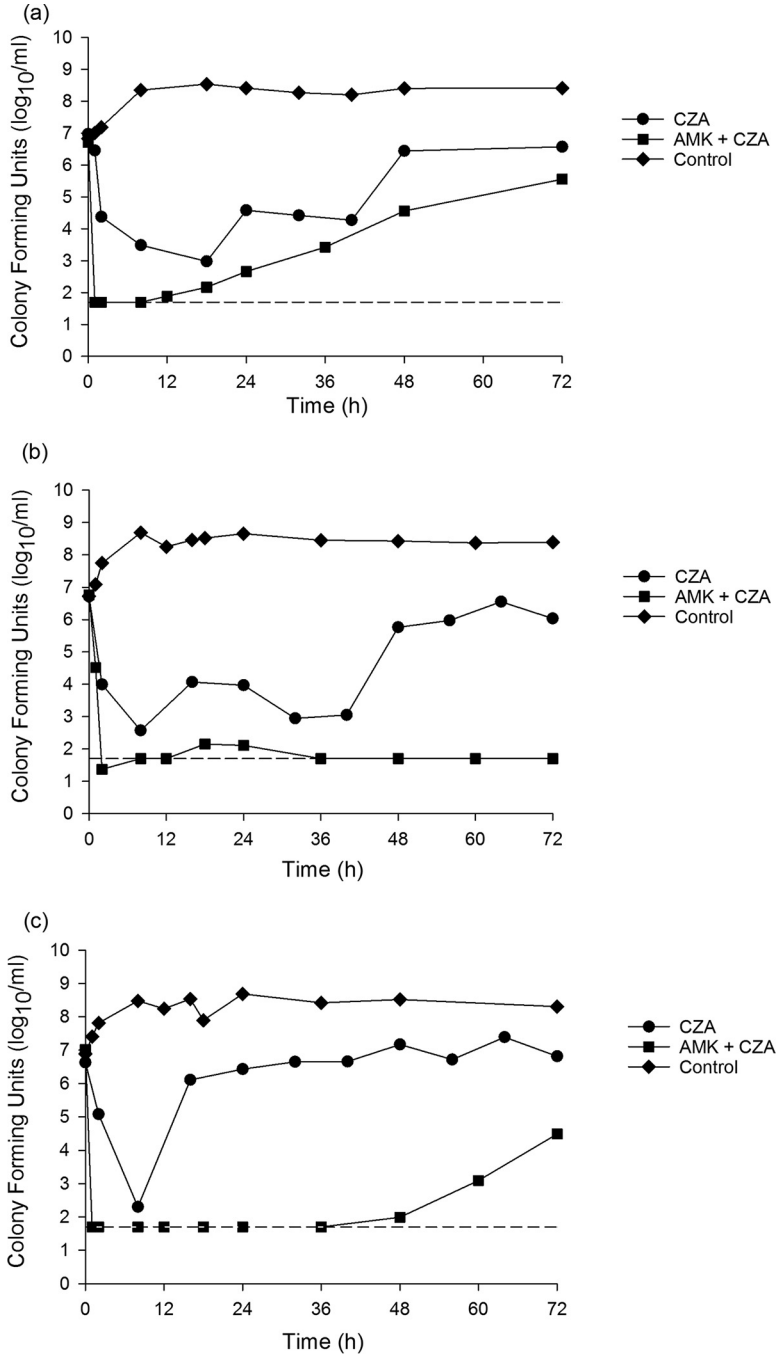

FIG 3 Mean bacterial densities over 72 h for ceftazidime-avibactam (CZA) alone and CZA plus amikacin (AMK) combination therapy against *K. pneumoniae* 329b (CZA MIC, 1 μg/ml; AMK MIC, 32 μg/ml) (a), *K. pneumoniae* 375 (CZA MIC, 4 μg/ml; AMK MIC, 64 μg/ml) (b), and *K. pneumoniae* 352 (CZA MIC, 8 μg/ml; AMK MIC, 64 μg/ml) (c). Dashed line, lower limit of detection.

for *P. aeruginosa*, 72-h CFU results did not directly follow with baseline MIC. For example, despite the lowest MIC (1/4 μg/ml), *K. pneumoniae* 329b regrew to 5.52 log_{10} CFU/ml over 72 h. This isolate had multiple genes encoding beta-lactamases in its genome, including those for SHV-11, SHV-5, OXA-9, TEM-1, and a KPC-2. Given the high $fT_{>MIC}$ achieved, it is not clear why this organism did not respond to ceftazidime-avibactam monotherapy. It was also interesting that despite the lower amikacin MIC (32 μg/ml) than for the other *Klebsiella* isolates, the combination regimen did not significantly reduce 72-h CFU values against this isolate (Fig. 3a; Table 3). In contrast, significant reductions following combination therapy

TABLE 3 CFU at 72 h for control, ceftazidime-avibactam alone, and ceftazidime-avibactam plus amikacin combination therapy

Isolate	CFU at 72 h (log_{10}/ml)[a]			*P* value (all groups)	*P* value (CZA vs CZA-amikacin)
	Control	CZA alone	CZA-amikacin combination		
P. aeruginosa					
C42-72	7.44 ± 0.21	6.80 ± 0.97	2.65 ± 1.35	0.017	0.027
C42-45	6.60 ± 0.24	5.61 ± 0.70	4.19 ± 0.34	0.005	0.020
1504	7.20 ± 0.52	4.36 ± 0.15	3.96 ± 0.01	0.003	0.298
K. pneumoniae					
329b	8.41 ± 0.14	5.52 ± 1.22[b]	5.56 ± 0.50	0.011	0.963
375	8.39 ± 0.29	6.04 ± 0.03	1.7 ± 0[c]	<0.001	<0.001
352	8.38 ± 0.23	6.82 ± 0.93	4.48 ± 0.30[d]	0.003	0.021

[a]Data are mean ± standard deviation of results from experiments in duplicate, unless otherwise noted. CZA, ceftazidime-avibactam.
[b]Mean ± standard deviation from four experiments with CZA alone against *K. pneumoniae* 329b.
[c]Both experiments reached the lower limit of detection (1.7 log_{10}/ml), so the standard deviation is zero.
[d]Mean ± standard deviation of results from three experiments with CZA-amikacin against *K. pneumoniae* 352.

were observed against *K. pneumoniae* 375 and 352, both of which produced the KPC-3 enzyme. To our knowledge, our data are the first to assess the effect of human simulated exposures of ceftazidime-avibactam plus amikacin combination therapy on KPC-producing *Klebsiella* spp.; however, others have reported *in vitro* synergistic interactions and improved survival in experimental infection models for this same regimen (24, 25).

Analyses of AUBC results (Table 4) further support observations at 72 h. In all experiments, the addition of amikacin significantly reduced the AUBC compared with ceftazidime-avibactam alone. As previously mentioned, a substantial difference in the curves can be visualized early in the first 12 to 24 h of the experiment, where the ceftazidime-avibactam/amikacin combination quickly reduced the inoculum to the lower limit of detection against five of the six isolates (Fig. 2 and 3). Although the *in vitro* pharmacodynamic chemostat model has no granulocytes, these observations have potential clinical relevance. Drusano and colleagues (26) have demonstrated the supportive role of granulocytes in pneumonia models when the inoculum can be rapidly reduced by at least 2 log_{10} CFU/g using optimal antibiotic therapy. Given that the pathogenic inoculum of bacterial pneumonia is ~6 log_{10} CFU/ml in humans (27), rapid reductions to less than 10^4 should enable a competent immune system to effectively clear pulmonary infection.

A notable observation during the experiment was the development of ceftazidime-avibactam resistance in *K. pneumoniae* 352, an isolate that produced KPC-3 as well as SHV-11. The MIC increased from 8/4 to 64/4 μg/ml within 24 h of exposure to

TABLE 4 AUBC over 72 h for control, ceftazidime-avibactam alone, and ceftazidime-avibactam plus amikacin combination therapy

Isolate	AUBC over 72 h (log_{10} · h/ml)[a]			*P* value (all groups)	*P* value (CZA vs CZA-amikacin)
	Control	CZA alone	CZA-amikacin combination		
P. aeruginosa					
C42-72	506 ± 12	394 ± 0.2	139 ± 21	<0.001	<0.001
C42-45	500 ± 7	359 ± 22	288 ± 3	0.001	0.013
1504	562 ± 34	301 ± 3	210 ± 8	<0.001	0.020
K. pneumoniae					
329b	592 ± 4	324 ± 23[b]	236 ± 28	<0.001	<0.001
375	605 ± 2	323 ± 64	133 ± 11	0.002	0.015
352	567 ± 56	447 ± 46	165 ± 6[c]	<0.001	0.001

[a]Data are mean ± standard deviation of results from experiments in duplicate, unless otherwise noted. AUBC, area under the bacterial growth curve; CZA, ceftazidime-avibactam.
[b]Mean ± standard deviation from four experiments with CZA alone against *K. pneumoniae* 329b.
[c]Mean ± standard deviation from three experiments with CZA-amikacin against *K. pneumoniae* 352.

ceftazidime-avibactam monotherapy. Resistance was stable and recapitulated every 24 h throughout the 72-h experiment. Ceftazidime-avibactam resistance development in the presence of plasmid-borne mutations in bla_{KPC-3} has been described in the literature (19, 20); furthermore, this resistance development has resulted in clinical failures. Although not confirmed in the current study, we speculate that the observed resistance could be due to mutations in the OmpK36 porin gene or in the omega loop of bla_{KPC-3} or by increased expression of KPC-3, all of which are possible after exposure to ceftazidime-avibactam (19, 20, 28). Combination therapy with exposures achieved by Amikacin Inhale resulted in resistance suppression over the 72-h experiment, despite this isolate having an amikacin MIC of 64 μg/ml. The suppression of resistance development with combination therapy has been observed in other *in vitro* and *in vivo* pharmacodynamic studies (29, 30).

Our study has some limitations which should be noted. First, this was an *in vitro* study, and therefore the contribution of an immune system cannot be accounted for. Second, the experiment was conducted for only up to 72 h, while antibiotic therapy is frequently provided for much longer. However, 72 h has been sufficient in other *in vitro* pharmacodynamic studies to provide preliminary benefits of combination therapy that should be further evaluated in *in vivo* infection models or clinical trials (29, 30). Finally, we used the pharmaceutical formulation of ceftazidime-avibactam that has a fixed 4:1 ratio for the chemostat experiments; therefore, avibactam concentrations were not measured but were assumed to be one-fourth that of the observed ceftazidime concentrations in the models. Based on its pharmacokinetics in ELF of healthy volunteers, avibactam was observed to have a peak concentration at 2 h that was at approximately this same ratio; however, in comparison with ceftazidime, the avibactam half-life was shorter in ELF, resulting in a lower AUC (13). We estimated that the ELF exposure for avibactam would be approximately 75% $fT_{>1\ \mu g/ml}$, while calculated exposures in the model were higher, at ~99%. Most importantly, all chemostat experiments had avibactam exposures greater than 50% $fT_{>1\ \mu g/ml}$, which is the maximal exposure required for the inhibition of beta-lactamases (31).

In summary, we observed significant reductions in CFU values against four of six carbapenem-resistant *P. aeruginosa* and *K. pneumoniae* isolates when exposures achieved with inhaled amikacin were combined with intravenous ceftazidime-avibactam in this *in vitro* pharmacodynamic model. Moreover, amikacin was successful in suppressing the emergence of ceftazidime-avibactam resistance in a *K. pneumoniae* isolate producing KPC-3. These observations suggest a beneficial role of Amikacin Inhale in combination with ceftazidime-avibactam against carbapenem-resistant *P. aeruginosa* and *K. pneumoniae* and warrant further investigation of this combination.

MATERIALS AND METHODS

Bacterial strains and MIC testing. Three clinical *P. aeruginosa* isolates (*P. aeruginosa* C42-72, C42-45, and 1504) and three clinical *K. pneumoniae* isolates (*K. pneumoniae* 329b, 375, and 352) were used in this study. The isolates were selected based on their carbapenem resistance profile, ceftazidime-avibactam MICs that were near the susceptibility breakpoint, and, finally, a wide range of amikacin MICs (8 to 64 μg/ml) that included both susceptible and nonsusceptible strains. To determine ceftazidime-avibactam and amikacin MICs, Clinical and Laboratory Standards Institute (CLSI) broth microdilution methods were used (32). Briefly, a Biomek 3000 instrument (Beckman Instruments, Inc., Fullerton, CA) was used to prepare MIC trays in-house and stored at −80°C until use. To dilute the MIC trays, cation-adjusted Mueller-Hinton broth (CAMHB) was obtained from Becton, Dickinson and Co. (Sparks, MD). Avibactam powder was purchased from Tecoland Corporation (Irvine, CA), and trays were prepared with a fixed concentration of 4 μg/ml; ceftazidime and amikacin powders were purchased from Sigma (St. Louis, MO). For each isolate, MICs were determined in triplicate on the same day, and the modal MIC was used to represent the final MIC. *Klebsiella pneumoniae* ATCC 700603 and *Pseudomonas aeruginosa* ATCC 27853 were used for quality control testing as recommended by the CLSI (32). Whole-genome sequencing of four out of six of the strains at baseline was performed at Walter Reed Army Institute of Research (Silver Spring, MD), as previously described (33, 34).

Antibiotics. Amikacin (BAY41-6551; lot number BXR774K, expiration date 26 June 2020), formulated for delivery via inhalation, was obtained from Bayer Pharma AG (Berlin, Germany). Ceftazidime-avibactam commercially available vials (Avycaz, lot number Q309, expiration date October 2019; Allergan Inc., Jersey City, NJ) were obtained from Cardinal Health (Dublin, OH). These formulations were used for all *in vitro*

pharmacodynamic chemostat experiments. Prior to each experiment, antibiotic stock solutions were prepared according to the pharmaceutical instructions.

Simulated drug exposures. The mean steady-state ELF concentrations of amikacin 400 mg q12h delivered via PDDS from critically ill patients were simulated in the *in vitro* pharmacodynamic model with target free peak and trough concentrations of 5,251 and 507 μg/ml, respectively, resulting in a free 12-h area under the curve (fAUC$_{0-12}$) exposure of 17,940 μg · h/ml (15, 16, 18). The mean steady-state ELF concentrations of ceftazidime-avibactam 2.5 g (2 g ceftazidime/0.5 g avibactam) every 8 h after a 2-h infusion from healthy adult volunteers undergoing bronchoalveolar lavage (BAL) were simulated with target free ceftazidime peak and trough concentrations of 23.2 and 7.8 μg/ml, respectively (13). Avibactam concentrations were assumed to follow those of ceftazidime in the model at precisely one-fourth the concentration, since ELF concentrations at the peak were proportional to the ceftazidime-avibactam formulation administered.

***In vitro* pharmacodynamic model.** A one-compartment *in vitro* chemostat model was used for all experiments. Briefly, each experiment consisted of three independent glass reactor models, two of them containing experimental treatments conducted in duplicate and one antibiotic-free growth control. All experiments were performed simultaneously for each isolate and treatment. Reactors were placed in a water bath with a temperature modulator set for 35°C, and magnetic stirrers were placed inside to ensure consistent mixing. All reactors were filled with 300 ml of CAMHB (Becton, Dickinson and Co., Sparks, MD). Thirty minutes prior to antibiotic(s) administration, the reactors were inoculated with 10^6 $\log_{10}$ CFU/ml of each isolate to allow bacteria to enter the log phase of growth. This inoculum simulated that observed in the ELF of patients with ventilator-associated pneumonia during clinical trials (27). After administration of the bolus of the antibiotic(s) at 0 h to achieve the target free peak concentration of amikacin and trough concentration of ceftazidime-avibactam, ceftazidime-avibactam was infused into each reactor over 2 h to achieve the target peak concentration via peristaltic pumps (Masterflex model 7519-05 and Masterflex L/S model 7519-15; Cole-Palmer Instrument Company, Vernon Hills, IL). Subsequently, fresh broth was infused into the models to simultaneously dilute the ceftazidime-avibactam and amikacin concentrations. The pump rate was set for a distributional half-life of amikacin over 3 h every 12 h, followed by the elimination half-life to achieve the target fAUC$_{0-12}$. Ceftazidime-avibactam supplemental vessels were used to adjust the concentrations infused every 8 h to achieve the correct ceftazidime and avibactam peaks at 2 h. Each experiment was conducted over 72 h.

To assess bacterial density over time, samples were taken from each reactor at a series of time points over the 72-h experiments and serially diluted in normal saline. Diluted samples were immediately plated into Trypticase soy agar with 5% sheep blood plates (BBL Stacker plate, 90-mm diameter; Becton, Dickinson and Co., Sparks, MD) and incubated at 37°C for 16 to 24 h, after which the colony counts were read. The lower limit of quantification was 1.7 $\log_{10}$ CFU/ml.

Antibiotic concentrations and exposure determination. Antibiotic concentrations obtained inside the reactors were measured at established time points over 72 h, with at least two concentrations measured within each interval rate change. All samples were immediately stored at −80°C until analysis. Amikacin samples were analyzed by Quest Diagnostics (Chantilly, PA, USA) using an enzyme multiple immunoassay technique (EMIT; AU680 analyzer using the Syva 2000 reagent kit from Beckman Coulter). The only modification of the methodology involved dilution of samples in CAMHB to a detectable concentration range of 2.5 to 50 μg/ml. The concentration of the ceftazidime component of ceftazidime-avibactam was assessed by a high-performance liquid chromatography (HPLC) method at the Center for Anti-Infective Research and Development (Hartford, CT, USA). The ceftazidime assay was linear over concentrations of 0.5 to 50 μg/ml. The mean intraday coefficients of variation (CVs) for low (1 μg/ml) and high (40 μg/ml) quality control samples were 4.57% and 2.10%, respectively. For the interday quality control samples, the CVs were 3.69% and 4.78%, respectively. Since the ratio of avibactam to ceftazidime in the pharmaceutical formulation was proportional to the concentrations observed in the ELF study, it was assumed that avibactam concentrations were one-fourth that of observed ceftazidime concentrations in the model. The fAUC$_{0-12}$ was calculated for amikacin using the log trapezoidal rule. Observed versus predicted concentrations of ceftazidime and amikacin were assessed via linear regression (SigmaPlot version 13.0; Systat Software Inc., San Jose, CA). Observed fAUC/MIC, $fT_{>MIC}$, and $fT_{>1\ \mu g/ml}$ were reported for amikacin, ceftazidime, and avibactam, respectively, noting once again that the avibactam concentrations were not measured directly.

Resistance emergence. Ceftazidime-avibactam and amikacin MICs were determined repeatedly, at 24, 48, and 72 h, during all chemostat experiments using broth microdilution. The organism inoculum was prepared directly from the plates providing CFU data for the *in vitro* pharmacodynamic studies to avoid loss of any unstable resistance mutations. Drug-free Trypticase soy agar with 5% sheep blood plates (BBL Stacker plate, 90-mm diameter; Beckton, Dickinson & Co., Sparks, MD) were used in all studies performed. MIC increases of at least 2 dilutions from the baseline values were defined as resistance emergence, noting that some of the selected isolates were already nonsusceptible to amikacin.

Statistical analyses. Bacterial density was measured by $\log_{10}$ CFU/ml over the 72-h experiment. Time-kill curves were constructed by plotting the mean bacterial density (CFU/ml) for each isolate and regimen against time. The primary endpoints for the study were the comparative observed $\log_{10}$ CFU/ml values at 72 h between controls, ceftazidime-avibactam alone, and the ceftazidime-avibactam plus amikacin combination. Secondary endpoints were the comparative overall area under the bacterial growth curves (AUBC) over the 72-h experiments. AUBC was calculated by the trapezoidal rule. Differences in the 72-h CFU/ml and the AUBC for each isolate were assessed by analysis of variance (ANOVA) with the Holm-Sidak method for multiple comparisons using SigmaPlot version 13.0. Statistical significance was defined *a priori* as a P value of <0.05.

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