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Amikacin-Fosfomycin at a Five-to-Two Ratio: Characterization of Mutation Rates in Microbial Strains Causing Ventilator-Associated Pneumonia and Interactions with Commonly Used Antibiotics

A. Bruce Montgomery,[a] Paul R. Rhomberg,[b] Tammy Abuan,[a] Kathie-Anne Walters,[a] Robert K. Flamm[b]

Cardeas Pharma, Seattle, Washington, USA[a]; JMI Laboratories, North Liberty, Iowa, USA[b]

The amikacin-fosfomycin inhalation system (AFIS), a combination of antibiotics administered with an in-line nebulizer delivery system, is being developed for adjunctive treatment of ventilator-associated pneumonia (VAP). The *in vitro* characterization of amikacin-fosfomycin (at a 5:2 ratio) described here included determining resistance selection rates for pathogens that are representative of those commonly associated with VAP (including multidrug-resistant strains) and evaluating interactions with antibiotics commonly used intravenously to treat VAP. Spontaneous resistance to amikacin-fosfomycin (5:2) was not observed for most strains tested (*n*, 10/14). Four strains had spontaneously resistant colonies (frequencies, 4.25×10^{-8} to 3.47×10^{-10}), for which amikacin-fosfomycin (5:2) MICs were 2- to 8-fold higher than those for the original strains. After 7 days of serial passage, resistance (>4-fold increase over the baseline MIC) occurred in fewer strains (*n*, 4/14) passaged in the presence of amikacin-fosfomycin (5:2) than with either amikacin (*n*, 7/14) or fosfomycin (*n*, 12/14) alone. Interactions between amikacin-fosfomycin (5:2) and 10 comparator antibiotics in checkerboard testing against 30 different Gram-positive or Gram-negative bacterial strains were synergistic (fractional inhibitory concentration [FIC] index, ≤0.5) for 6.7% (*n*, 10/150) of combinations tested. No antagonism was observed. Synergy was confirmed by time-kill methodology for amikacin-fosfomycin (5:2) plus cefepime (against *Escherichia coli*), aztreonam (against *Pseudomonas aeruginosa*), daptomycin (against *Enterococcus faecalis*), and azithromycin (against *Staphylococcus aureus*). Amikacin-fosfomycin (5:2) was bactericidal at 4-fold the MIC for 7 strains tested. The reduced incidence of development of resistance to amikacin-fosfomycin (5:2) compared with that for amikacin or fosfomycin alone, and the lack of negative interactions with commonly used intravenous antibiotics, further supports the development of AFIS for the treatment of VAP.

Ventilator-associated pneumonia (VAP) is a nosocomial infection that develops more than 48 h following intubation for mechanical ventilation (1). Reports indicate that as many as 50% of mechanically ventilated patients are infected with more than one pathogen (2–4). Approximately 80% of cases of pneumonia among mechanically ventilated patients involve *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella* spp., *Escherichia coli*, *Acinetobacter* spp., and/or *Enterobacter* spp. Other pathogens, including *Serratia* spp., *Stenotrophomonas maltophilia*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*, may also be involved. Definitive microbiological culture results require as long as 3 days to obtain, and the more rapidly obtained Gram stains of tracheal secretions lack sensitivity and specificity (5–7).

The standard treatment for VAP is intravenous (i.v.) antibiotics. Inappropriate empirical therapy during the first 48 h, even when followed by microbiologically directed therapy, has been associated with approximately 90% mortality among mechanically ventilated patients (8). Consequently, the choice of an initial antibiotic is crucial and must account for a range of organisms, including highly resistant Gram-negative pathogens and the possible presence of methicillin-resistant *S. aureus* (MRSA). Currently available i.v. antibiotics may not effectively treat this wide range of bacteria with a safety profile that is acceptable for widespread empirical use while one is waiting for culture verification.

Further, i.v. antibiotics exhibit uneven distribution in the airways and poor penetration into epithelial fluids, often resulting in subtherapeutic concentrations at the site of infection, despite high, and potentially toxic, systemic concentrations (9). Exposure to subtherapeutic concentrations of antibiotics at the site of infection contributes, theoretically at least, to the selection of resistant bacterial strains. The increasing frequencies of Gram-negative bacteria that produce β-lactamases (e.g., *Klebsiella pneumoniae* carbapenemase [KPC]) and of multidrug-resistant (MDR) Gram-negative bacteria, and the increasingly widespread occurrence of MRSA, are continuously eroding the utility of i.v. antibiotics in the treatment of VAP and are cause for worldwide concern (10–12). Few new classes of i.v. antibiotics are under development, and the failure rate of new drugs in clinical trials is high, so there is little near-term hope for multiple new classes of drugs for treating such drug-resistant bacteria.

Delivery of antibiotics directly to the airways is a proven approach that offers several key advantages over a maximum i.v. dose, including higher concentrations in sputum and reduced systemic exposure (13). Additionally, antibiotics are rapidly cleared from the lung, so that despite maximum concentrations that can be well above the MIC, trough concentrations do not persist at sub-MIC levels, thus reducing the risk of treatment-emergent resistance. In addition, the combination of amikacin and fosfomy-

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Address correspondence to A. Bruce Montgomery, Bruce.Montgomery@CardeasPharma.com.

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cin has been shown to decrease the frequency of spontaneous resistance (14). Aerosolized antibiotics have been studied in small clinical trials in patients with VAP (15–17). The results of these studies suggest that treatment of VAP with adjunctive aerosolized antibiotics may reduce the frequency of emergence of antibiotic-resistant bacteria. For example, in a study of VAP caused by *P. aeruginosa*, treatment with nebulized ceftazidime and amikacin alone was noninferior to treatment with i.v. antibiotics alone, and the emergence of antibiotic-resistant *P. aeruginosa* was observed after i.v. therapy, but not after aerosolized therapy (16).

The amikacin-fosfomycin inhalation system (AFIS) is a combination of 2 antibiotics administered with an in-line nebulizer delivery system that is being developed for the adjunctive treatment of VAP, more specifically, to treat patients with pneumonia caused by Gram-negative organisms, as well as those coinfected with Gram-positive bacteria (18). AFIS consists of 300 mg amikacin and 120 mg fosfomycin in a total of 6 ml, administered in standard ventilator circuits with the PARI Investigational eFlow Inline System. In a phase 1 study, high airway antibiotic concentrations were achieved with AFIS (median amikacin concentration, 11,400 μg/g sputum; median fosfomycin concentration, 6,650 μg/g sputum) (18).

In a previous *in vitro* study, presented in our accompanying article (19), combining amikacin with fosfomycin at a 5:2 ratio significantly enhanced the potency of amikacin against 62 amikacin-nonsusceptible Gram-negative pathogens. All amikacin-fosfomycin MIC results were ≤256 and ≤102.4 μg/ml, respectively, and MIC values for the combination of antibiotics were lower than those of amikacin alone (MIC range, 32 to >1,024 μg/ml) or fosfomycin alone (MIC range, 3.2 to 204.8 μg/ml). Interactions between amikacin and fosfomycin differed by isolate, ranging from no detectable interaction to high synergy. Comparison of the *in vitro* MIC results with the high airway antibiotic concentrations achieved *in vivo* reveals that AFIS is well positioned to achieve the goal of delivering a broad-spectrum fixed-dose antibiotic combination at concentrations that will be effective in treating highly resistant bacteria, thus limiting the selection of resistant strains.

The *in vitro* activity of amikacin-fosfomycin (at a 5:2 ratio) was further characterized in the studies described here. Amikacin-fosfomycin (5:2) resistance selection rates were determined for pathogens that were representative of those commonly associated with VAP, including MDR bacterial strains, by using single-step spontaneous and serial passaging mutational analyses. Checkerboard synergy and time-kill interaction studies were conducted to confirm that no adverse interactions occurred between amikacin-fosfomycin (5:2) and antibiotics commonly used in the i.v. treatment of VAP.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains were either obtained from ATCC (Manassas, VA) or selected from a worldwide antimicrobial surveillance collection (SENTRY), which contains a library of >35,000 organisms collected in 2011 from >100 medical centers on 6 continents (JMI Laboratories, North Liberty, IA).

Single-step (spontaneous) and serial passaging mutational analyses. (i) Strains. Fourteen bacterial strains were evaluated, including 3 MDR clinical isolates of *Acinetobacter* spp., 1 strain of *Escherichia coli* (ATCC 25922), 1 clinical isolate of *Enterobacter cloacae*, 3 clinical isolates of *Klebsiella pneumoniae* (2 of which produced extended-spectrum β-lactamases [ESBL], while 1 produced KPC β-lactamase), 3 strains of *Pseudomonas aeruginosa* (ATCC 27853 and 2 carbapenem-resistant clinical isolates), 2 strains of *Staphylococcus aureus* (1 methicillin-susceptible *S. aureus* [MSSA] strain [ATCC 29213] and 1 methicillin-resistant *S. aureus* [MRSA] clinical isolate), and 1 strain of *Streptococcus pneumoniae* (ATCC 49619).

(ii) Baseline MICs. Baseline amikacin and fosfomycin MIC values for the amikacin-fosfomycin (5:2) combination were determined for each bacterial strain using reference agar dilution methods (20). Serial passage studies were performed in a broth microdilution format, and susceptibility was interpreted using CLSI criteria (21). Testing (either agar dilution or broth microdilution) was performed in cation-adjusted Mueller-Hinton broth medium with 25 μg/ml glucose-6-phosphate.

(iii) Single-step (spontaneous) mutation rates. Fresh colonies from an agar plate were suspended in sterile water until at least a 4 McFarland standard was achieved (approximately 1×10^9 to 2×10^9 CFU/ml), and 0.1 ml and 1.0 ml of each suspension were plated on agar plates containing amikacin-fosfomycin (5:2) at concentrations that were 4-fold, 8-fold, and 16-fold higher than the baseline MIC for each strain. Tests were performed in duplicate. Serial dilutions were plated on antibiotic-free agar plates in order to determine the CFU/ml for each initial bacterial suspension. Resistance rates were calculated by dividing the number of resistant mutant colonies (CFU/ml) that grew on antibiotic-containing agar plates by the number of CFU/ml in the initial antibiotic-free starting inoculum (approximately 10^9 CFU/ml). If resistant colonies were observed, representative colonies (≤10 per strain) were selected and were retested against amikacin-fosfomycin (5:2) using reference agar dilution and broth microdilution methods (20) in order to confirm the resistance.

(iv) Serial-passaging mutational analysis. To measure the stepwise development of resistance, strains were passaged by using a broth microdilution method over a period of 7 days in the presence of serial 2-fold dilutions of amikacin-fosfomycin (5:2), amikacin, or fosfomycin. MIC values at baseline were compared to those at day 7 (agar dilution method). To assess the stability of resistance, organisms for which the MIC increased during serial passage in the presence of an antibiotic were transferred from the broth well that showed growth (highest antibiotic concentration) directly to antibiotic-free sheep blood agar plates. MIC values were then determined from growth on the sheep blood agar plates by broth microdilution. Those organisms that were confirmed as resistant were then passaged twice on antibiotic-free sheep blood agar plates. MICs for these subcultured strains were determined by broth microdilution in order to ascertain if there was a reversion of the MIC.

Interaction of amikacin-fosfomycin (5:2) with other antibiotics. (i) Strains. A total of 30 organisms were tested. The 15 Gram-positive organisms included *Staphylococcus aureus* (10 strains: ATCC 29213, 1 MSSA clinical isolate, and 8 MRSA clinical isolates), *Enterococcus faecalis* (2 strains: ATCC 29212 and 1 clinical isolate), and *S. pneumoniae* (3 strains: ATCC 49619 and 2 clinical isolates). The 15 Gram-negative organisms included *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (4 strains: 3 clinical isolates with the ESBL phenotype and 1 with the KPC phenotype), *Enterobacter cloacae* (4 clinical isolates), *Pseudomonas aeruginosa* (3 strains, comprising ATCC 27853 and 2 carbapenem-resistant clinical isolates), and *Acinetobacter* spp. (3 clinical isolates).

(ii) Baseline MICs. Baseline MIC testing was performed in 96-well panels, under standard CLSI broth microdilution testing conditions (cation-adjusted Mueller-Hinton broth, 35°C, ambient atmosphere, 16 to 20 h of incubation) in conformance with CLSI standards (20).

(iii) Checkerboard synergy testing. Broth microdilution was performed in a checkerboard configuration in a 96-well panel, in conformance with established procedures (22–25). Ninety-six-well panels were configured with serial dilutions of amikacin-fosfomycin (5:2) and were paired with serial dilutions of gentamicin, aztreonam, cefepime, meropenem, or tigecycline for Gram-negative bacilli or with azithromycin, vancomycin, daptomycin, linezolid, or tigecycline for Gram-positive cocci. Panels were produced at JMI Laboratories (North Liberty, IA). Antibiotics were tested by serial 2-fold dilutions (11 dilutions for amikacin-fosfomycin [5:2]; 7 dilutions for comparator antibiotics), spanning

TABLE 1 Frequency of spontaneous mutations among 14 strains tested at 4-, 8-, and 16-fold the baseline MIC for amikacin-fosfomycin (5:2)

Species	Phenotype[a]	Strain no.	Baseline MIC (μg/ml) of each drug in the amikacin-fosfomycin (5:2) combination		Mutation frequency[b] at:		
			Amikacin	Fosfomycin	4× Baseline MIC	8× Baseline MIC	16× Baseline MIC
Acinetobacter sp.	MDR	1986	4	1.6	$<1.65 \times 10^{-9}$	$<1.65 \times 10^{-9}$	$<1.65 \times 10^{-9}$
	MDR	8133	32	12.8	$<1.52 \times 10^{-11}$	$<1.52 \times 10^{-11}$	$<1.52 \times 10^{-11}$
	MDR	8572	128	51.2	$<9.35 \times 10^{-12}$	$<9.35 \times 10^{-12}$	$<9.35 \times 10^{-12}$
E. coli	Wild type	ATCC 25922	1	0.4	$<1.23 \times 10^{-12}$	$<1.23 \times 10^{-12}$	$<1.23 \times 10^{-12}$
E. cloacae	AmpC	5686	8	3.2	4.25×10^{-8}	$<7.69 \times 10^{-11}$	$<7.69 \times 10^{-11}$
K. pneumoniae	KPC	25	8	3.2	1.63×10^{-9}	$<1.41 \times 10^{-11}$	$<1.41 \times 10^{-11}$
	ESBL	341	1	0.4	$<1.74 \times 10^{-12}$	$<1.74 \times 10^{-12}$	$<1.74 \times 10^{-12}$
	ESBL	1015	8	3.2	2.28×10^{-9}	$<1.18 \times 10^{-11}$	$<1.18 \times 10^{-11}$
P. aeruginosa	Wild type	ATCC 27853	2	0.8	$<1.30 \times 10^{-11}$	$<1.30 \times 10^{-11}$	$<1.30 \times 10^{-11}$
	Carb-R	1113	8	3.2	$<1.27 \times 10^{-11}$	$<1.27 \times 10^{-11}$	$<1.27 \times 10^{-11}$
	Carb-R	1185	8	3.2	3.47×10^{-10}	$<1.36 \times 10^{-12}$	$<1.36 \times 10^{-12}$
S. aureus	MSSA	ATCC 29213	1	0.4	$<1.74 \times 10^{-12}$	$<1.74 \times 10^{-12}$	$<1.74 \times 10^{-12}$
	MRSA	70	8	3.2	$<8.13 \times 10^{-12}$	$<8.13 \times 10^{-12}$	$<8.13 \times 10^{-12}$
S. pneumoniae	Pen-I	ATCC 49619	16	6.4	$<1.60 \times 10^{-12}$	$<1.60 \times 10^{-12}$	$<1.60 \times 10^{-12}$

[a] AmpC, AmpC type β-lactamase; Carb-R, carbapenem resistant; ESBL, extended-spectrum β-lactamase; KPC, *Klebsiella pneumoniae* carbapenemase; MDR, multidrug resistant; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-sensitive *S. aureus*; Pen-I, penicillin intermediate.

[b] Calculated as the number of resistant mutant colonies (CFU/ml) that grew on antibiotic-containing agar plates divided by the number of CFU/ml in the initial antibiotic-free starting inoculum (approximately 10^9 CFU/ml).

the range of 0.25-fold to 2-fold the baseline MIC value for each test organism.

The fractional inhibitory concentration (FIC) for amikacin-fosfomycin (5:2) (agent 1) in combination with a second antibiotic (agent 1 in combination with agent 2) was calculated as the MIC of agent 1 in combination with agent 2, divided by the MIC of agent 1 alone. The FIC for agent 2 was calculated as the MIC of agent 2 in combination with agent 1, divided by the MIC of agent 2 alone. A FIC index was calculated for the combination of agents 1 and 2 as the sum of the individual FICs of agent 1 and agent 2. Synergy was defined as an FIC index of ≤0.5, indifference as >0.5 to ≤4.0, and antagonism as >4.0.

(iv) Time-kill synergy testing. Antibiotic-strain combinations with checkerboard synergy testing results indicating synergy were further tested by time-kill assays. Amikacin-fosfomycin (5:2) and the comparator antibiotics were each tested alone at concentrations corresponding to their MICs and were tested in combination, at an amikacin-fosfomycin (5:2) concentration that was equal to its MIC and a comparator antibiotic concentration that was 0.25-fold of its MIC. The amikacin-fosfomycin (5:2) combination alone was also tested at 4× MIC. Time-kill samples were plated for colony counts at 0, 2, 4, 8, and 24 h after initiation. Synergy was defined as a ≥2 $\log_{10}$-CFU/ml decrease in the colony count with the combination from that with the most efficient agent alone at 24 h. Indifference was defined as a decrease of ±1 $\log_{10}$ to <2 $\log_{10}$ CFU/ml with the combination compared to the most efficient agent alone, and antagonism was defined as an increase of >1 $\log_{10}$ CFU/ml with the combination compared to the less-active single agent alone. Bactericidal activity for an individual drug was defined as a ≥3 $\log_{10}$-CFU/ml (99.9%) reduction in the colony count at 24 h from that of the starting inoculum.

RESULTS

Single-step mutational analysis. Resistant colonies were observed for 4 of the 14 tested strains at amikacin-fosfomycin (5:2) concentrations that were 4-fold higher than the baseline amikacin-fosfomycin (5:2) MIC values (Table 1). No resistant colonies were observed for any of the 14 strains at concentrations 8-fold or 16-fold higher than the baseline MIC values. Mutation frequencies for the 4 strains with resistant colonies were 4.25×10^{-8} (*E. cloacae*), 1.63×10^{-9} and 2.28×10^{-9} (2 *K. pneumoniae* strains), and 3.47×10^{-10} (*P. aeruginosa*). Mutation frequencies for the other 10 strains were all $<1.27 \times 10^{-11}$ (except for *Acinetobacter* sp. strain 1986, for which the mutation frequency was $<1.65 \times 10^{-9}$).

Representative colonies from the 4 resistant strains were retested to confirm the observed resistance to amikacin-fosfomycin (5:2). Amikacin-fosfomycin (5:2) MIC values had increased over the baseline for each representative colony from the 4 resistant strains. Eight-fold increases from baseline amikacin-fosfomycin (5:2) MIC values were the largest increases observed and occurred for all 9 representative colonies from the resistant *E. cloacae* strain 5686 and for 2 of 10 representative colonies from the resistant *P. aeruginosa* strain 1185. Two- to 4-fold increases over baseline amikacin-fosfomycin (5:2) MIC values occurred for the remaining 8 representative colonies from the resistant *P. aeruginosa* strain 1185 and for 10 representative colonies from each of 2 resistant *K. pneumoniae* strains (strains 25 and 1015).

Serial-passaging mutational analysis. The 14 strains were passaged for 7 days in the presence of amikacin-fosfomycin (5:2), amikacin, or fosfomycin, and MIC values at day 7 were compared with baseline (day 1) MIC values. To determine if any changes in MIC values that were observed at day 7 were stable, the strains were then passaged twice on blood agar plates with no antibiotic, and MIC values were redetermined.

Resistance, defined as a >4-fold increase over the baseline MIC, was observed for fewer strains after serial passage in the

presence of amikacin-fosfomycin (5:2) than after serial passage in the presence of either amikacin or fosfomycin alone (Table 2). Resistance was observed for 4 of the 14 strains passaged in the presence of amikacin-fosfomycin (5:2), including 1 strain each of *E. coli*, *E. cloacae*, ESBL-producing *K. pneumoniae*, and *S. pneumoniae*, which showed increases in amikacin-fosfomycin (5:2) MIC values of 8-, 32-, 8-, and 16-fold, respectively (Table 2). After 2 passages on antimicrobial-free medium, elevated amikacin-fosfomycin (5:2) MIC values were retained for all 4 strains, with no changes observed for 3 strains and a 2-fold decrease in the amikacin-fosfomycin (5:2) MICs observed for *E. cloacae* (from 512 and 204.8 μg/ml to 256 and 102.4 μg/ml), which were still higher than the baseline amikacin-fosfomycin (5:2) MICs for this strain (16 and 6.4 μg/ml).

In comparison, after serial passage in amikacin alone, resistance was observed for 7 of the 14 strains, and a 64-fold increase in the amikacin MIC was the largest increase observed (Table 2). After 2 passages on antimicrobial-free medium, amikacin MIC values remained stable for 1 of the 7 strains, increased for 1 strain, and decreased for the other 5 strains but remained higher than the baseline amikacin MIC. After serial passage in fosfomycin alone, resistance was observed for 12 of the 14 strains (or possibly 13 strains; see Table 2, footnote *c*), and a 512-fold increase was the largest increase in the fosfomycin MIC observed. After 2 passages on antimicrobial-free medium, fosfomycin MIC values remained unchanged for 2 of the 12 strains and decreased for 10 strains but remained higher than the baseline fosfomycin MIC for 7 of the 10 strains.

Among all 14 strains, the highest amikacin-fosfomycin (5:2) MICs observed at day 7 after passage in amikacin-fosfomycin (5:2) were 512 and 204.8 μg/ml (*Acinetobacter* sp. 8572), with MIC values of >64 and >25.6 μg/ml observed for a total of 3 strains. In comparison, the highest amikacin MIC at day 7 after passage in amikacin alone was >1,024 μg/ml, observed for the same *Acinetobacter* sp. strain (strain 8572), with MIC values of >64 μg/ml observed for a total of 6 strains. The highest fosfomycin MIC at day 7 after passage in fosfomycin alone was >409.6 μg/ml, observed for 11 of the 14 strains, with MIC values of >25.6 μg/ml observed for all 14 strains.

Only 1 strain had spontaneously resistant colonies in the single-step mutational analysis and also had a >4-fold increase in the amikacin-fosfomycin MIC after 7 days of serial passage in amikacin-fosfomycin (5:2). *E. cloacae* 5686 had a spontaneous mutation frequency of 4.25×10^{-8} and had amikacin-fosfomycin (5:2) MICs that increased 32-fold over the baseline after 7 days of serial passage (from 16 and 6.4 μg/ml to 512 and 204.8 μg/ml). After 2 passages without antibiotics, the amikacin-fosfomycin (5:2) MICs decreased 2-fold for this strain, to 256 and 102.4 μg/ml.

Interaction of amikacin-fosfomycin (5:2) with other antibiotics (checkerboard FIC results). Combinations of amikacin-fosfomycin (5:2) and 10 comparator antibiotics were tested against a total of 30 strains that were representative of bacterial species commonly associated with VAP. No antagonism (FIC, >4) was observed (Table 3). Synergy (FIC, ≤0.5) was observed for 10 (6.7%) of the antibiotic-strain combinations (Tables 3 and 4).

Time-kill kinetics. Time-kill kinetics were assessed for 9 of the 10 different antibiotic-strain combinations that showed synergy with amikacin-fosfomycin (5:2) in checkerboard testing. Synergy was observed for 4 of the 9 antibiotic-strain combinations (Table 4).

TABLE 2 Broth microdilution serial passage for 7 days in the presence of amikacin-fosfomycin (5:2), amikacin, or fosfomycin, followed by 2 passages on sheep blood agar plates containing no antibiotics

Species	Phenotype[a]	Strain no.	Amikacin-fosfomycin (5:2)				Amikacin				Fosfomycin			
			MICs[b] (μg/ml) at:		Fold increase (day 7/day 1)	MICs[b] (μg/ml) after 2 antibiotic-free passages	MIC (μg/ml) at:		Fold increase (day 7/day 1)	MIC (μg/ml) after 2 antibiotic-free passages	MIC (μg/ml) at:		Fold increase (day 7/day 1)	MIC (μg/ml) after 2 antibiotic-free passages
			Day 1	Day 7			Day 1	Day 7			Day 1	Day 7		
Acinetobacter sp.	MDR	1986	4, 1.6	8, 3.2	2	8, 3.2	4	8	2	8	102.4	>409.6	>4	409.6
	MDR	8133	64, 25.6	64, 25.6	1	128, 51.2	64	512	8	128	102.4	>409.6	>4	51.2
	MDR	8572	128, 51.2	512, 204.8	4	512, 204.8	128	>1,024	>8	512	204.8	>409.6	>2[c]	>409.6
E. coli	Wild type	ATCC 25922	1, 0.4	8, 3.2	8	8, 3.2	2	32	16	8	0.8	409.6	512	204.8
E. cloacae	AmpC	5686	16, 6.4	512, 204.8	32	256, 102.4	32	256	8	512	25.6	>409.6	>16	204.8
K. pneumoniae	KPC	25	16, 6.4	32, 12.8	2	32, 12.8	32	512	16	64	12.8	>409.6	>32	102.4
	ESBL	341	1, 0.4	8, 3.2	8	8, 3.2	2	128	64	16	12.8	>409.6	>32	12.8
	ESBL	1015	16, 6.4	64, 25.6	4	64, 25.6	32	32	1	64	6.4	>409.6	>64	>409.6
P. aeruginosa	Wild type	ATCC 27853	2, 0.8	8, 3.2	4	4, 1.6	2	8	4	4	6.4	>409.6	>64	12.8
	Carb-R	1113	4, 1.6	8, 3.2	2	8, 3.2	8	8	1	8	25.6	51.2	2	25.6
	Carb-R	1185	8, 3.2	16, 6.4	2	16, 6.4	4	16	4	16	51.2	>409.6	>8	102.4
S. aureus	MSSA	ATCC 29213	1, 0.4	2, 0.8	2	2, 0.8	2	4	2	2	0.8	>409.6	>512	3.2
	MRSA	70	8, 3.2	32, 12.8	4	32, 12.8	8	64	8	64	12.8	>409.6	>32	51.2
S. pneumoniae	Pen-I	ATCC 49619	8, 3.2	128, 51.2	16	128, 51.2	32	128	4	128	6.4	204.8	32	204.8

[a] AmpC, AmpC type β-lactamase; Carb-R, carbapenem resistant; ESBL, extended-spectrum β-lactamase; KPC, *Klebsiella pneumoniae* carbapenemase; MDR, multidrug resistant; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-sensitive *S. aureus*; Pen-I, penicillin intermediate.

[b] Given as amikacin MIC, fosfomycin MIC.

[c] The >2-fold increase to >409.6 μg/ml could be a >4-fold increase, which would meet the definition of resistance (a >4-fold increase over the day 1 MIC).

TABLE 3 Summary of FIC index interpretation categories for amikacin-fosfomycin (5:2) with selected comparator antibiotics tested against 15 Gram-positive and 15 Gram-negative pathogens

Comparator antibiotic	No. of test results in the following FIC index interpretative category[a]:			
	Synergy (FIC, ≤0.5)	Indifference (FIC, >0.5 to ≤4)	Indeterminacy (FIC not interpretable)[b]	Antagonism (FIC, >4)
For Gram negative bacteria ($n = 15$)				
Aztreonam	2	7	6	0
Cefepime	2	9	4	0
Gentamicin	1	11	3	0
Meropenem	3	10	2	0
Tigecycline	0	11	4	0
For Gram-positive bacteria ($n = 15$)				
Azithromycin	1	3	11	0
Daptomycin	1	14	0	0
Linezolid	0	15	0	0
Tigecycline	0	14	1	0
Vancomycin	0	15	0	0
Total	10	109	31	0

[a] Among all antibiotic-strain combinations, 6.7% exhibited synergy, 72.7% exhibited indifference, 20.7% gave indeterminate results, and 0.0% exhibited antagonism.
[b] Thirty-one results were not interpretable due to off-scale MICs and were labeled indeterminate. Of these, 13 tests gave off-scale high MICs and 8 gave off-scale low MICs for the comparator agent; 2 gave off-scale high values and 7 gave off-scale low values for the amikacin-fosfomycin combination; and 1 gave off-scale low values for both the comparator agent and the amikacin-fosfomycin combination.

The other 5 antibiotic-strain combinations had time-kill kinetics that indicated indifference. For all 7 strains for which time-kill-kinetic analyses were performed, amikacin-fosfomycin (5:2) was bactericidal at 4-fold the MIC.

DISCUSSION

Analyses to evaluate the development of antibiotic resistance were conducted on 14 strains that are representative of bacterial species commonly associated with VAP. For 10 of the strains, no colonies with spontaneous resistance to amikacin-fosfomycin (5:2) were observed. Low levels of spontaneously resistant colonies were observed for the other 4 strains, for which amikacin-fosfomycin (5:2) MIC values were 2- to 8-fold higher than those for the original strains. After 7 days of serial passage, resistance (a >4-fold increase over the baseline MIC) occurred in fewer strains passaged in the presence of the amikacin-fosfomycin (5:2) combination (4 of 14 strains) than in strains serially passaged in either amikacin alone (7 of 14 strains) or fosfomycin alone (12 of 14 strains).

Antibiotic resistance develops with prolonged exposure to antibiotic levels that are below the MIC. In addition to the decreased resistance observed with the use of a combination of two antibiotics, as demonstrated by the results described here, the planned use of the amikacin-fosfomycin (5:2) combination as an aerosol will achieve higher concentrations in tracheal aspirates (sputum) than a maximum dose delivered i.v., with reduced systemic exposure (13). Large increases in the development of antibiotic resistance have not been observed in studies of other aerosolized antibiotics, including nebulized ceftazidime and amikacin for the treatment of VAP (16) and multiple 28-day courses of tobramycin for inhalation (TOBI) (26) or aztreonam for inhalation (AZLI) (27) for the treatment of patients with cystic fibrosis and *P. aeruginosa* airway infections.

Since the amikacin-fosfomycin (5:2) combination is planned for use as adjunctive therapy with standard i.v. treatments for pneumonia, it was important to test for antagonistic interactions between amikacin-fosfomycin (5:2) and other antibiotics commonly used for i.v. treatment of VAP. *In vitro* checkerboard testing was conducted to assess the antibiotic activity resulting from combining amikacin-fosfomycin (5:2) and 10 antibiotics. Testing was conducted using 30 different strains that were representative of

TABLE 4 Time-kill kinetics for antibiotic–bacterial strain combinations showing synergy[a] in checkerboard testing

Antibiotic-strain combination	Phenotype[b]	Strain no.	FIC index for amikacin-fosfomycin (5:2) plus comparator agent[c]	Time-kill kinetics result	
				Log_{10} reduction in CFU/ml at 24 h[d]	Interpretive category
For Gram-negative bacteria					
Aztreonam					
E. cloacae	AmpC	5686	0.5	1.7	Indifference
P. aeruginosa	Wild type	ATCC 27853	0.5	2.1	Synergy
Cefepime					
E. cloacae	AmpC	5686	0.375	<0.5	Indifference
E. coli	Wild type	ATCC 25922	0.5	>8	Synergy
Gentamicin–*Acinetobacter* sp.	MDR	8133	0.5	0.2 increase	Indifference
Meropenem					
Acinetobacter sp.	MDR	1986	0.5	0.8	Indifference
E. cloacae	AmpC	5686	0.5	1.3	Indifference
K. pneumoniae	KPC	25	0.375	ND	
For Gram-positive bacteria					
Azithromycin–*S. aureus*	MSSA	ATCC 29213	0.5	3.0	Synergy
Daptomycin–*E. faecalis*	Wild type	ATCC 29212	0.5	2.7	Synergy

[a] Defined as a FIC index of ≤0.5.
[b] AmpC, AmpC type β-lactamase; KPC, *Klebsiella pneumoniae* carbapenemase; MDR, multidrug resistant.
[c] Determined by checkerboard synergy testing.
[d] ND, not determined.

bacterial species commonly associated with VAP. The checkerboard testing results indicated that the interactions between amikacin-fosfomycin (5:2) and the comparator antibiotics against Gram-positive or Gram-negative bacteria were synergistic for 6.7% (*n*, 10/150) of the comparator antibiotic-strain combinations tested, and no antagonism was observed for any combination. The synergy observed in checkerboard testing was confirmed by time-kill methodology, which also demonstrated that amikacin-fosfomycin (5:2) was bactericidal at 4-fold the MIC for the 7 bacterial strains tested. Importantly, no antagonism was observed; thus, the use of this combination of aerosolized antibiotics would not be expected to alter the efficacy of the standard i.v. treatments now in use.

In summary, amikacin-fosfomycin (5:2) reduced the incidence of development of resistance after prolonged antibiotic exposure and reduced the magnitude of the increases in MIC values from the increases observed with amikacin or fosfomycin alone. No negative interactions with antibiotics commonly used for i.v. treatment of VAP were observed. These microbiological results further support the development of amikacin-fosfomycin (5:2) for aerosol treatment of pneumonia caused by Gram-negative bacteria in patients on mechanical ventilation.

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REFERENCES

1. **Koenig SM, Truwit JD.** 2006. Ventilator-associated pneumonia: diagnosis, treatment, and prevention. Clin. Microbiol. Rev. **19:**637–657. http://dx.doi.org/10.1128/CMR.00051-05.
2. **Jiménez P, Torres A, Rodriguez-Roisin R, de la Bellacasa JP, Aznar R, Gatell JM, Agustí-Vidal A.** 1989. Incidence and etiology of pneumonia acquired during mechanical ventilation. Crit. Care Med. **17:**882–885. http://dx.doi.org/10.1097/00003246-198909000-00007.
3. **Kollef MH, Silver P.** 1995. Ventilator-associated pneumonia: an update for clinicians. Respir. Care **40:**1130–1140.
4. **Rello J, Quintana E, Ausina V, Castella J, Luquin M, Net A, Prats G.** 1991. Incidence, etiology, and outcome of nosocomial pneumonia in mechanically ventilated patients. Chest **100:**439–444. http://dx.doi.org/10.1378/chest.100.2.439.
5. **Albert M, Friedrich JO, Adhikari NK, Day AG, Verdant C, Heyland DK; Canadian Critical Care Trials Group.** 2008. Utility of Gram stain in the clinical management of suspected ventilator-associated pneumonia. Secondary analysis of a multicenter randomized trial. J. Crit. Care **23:**74–81. http://dx.doi.org/10.1016/j.jcrc.2008.01.004.
6. **Goldberg AE, Malhotra AK, Riaz OJ, Aboutanos MB, Duane TM, Borchers CT, Martin N, Ivatury RR.** 2008. Predictive value of bronchoalveolar lavage fluid Gram's stain in the diagnosis of ventilator-associated pneumonia: a prospective study. J. Trauma **65:**871–876. http://dx.doi.org/10.1097/TA.0b013e31818481e0.
7. **O'Horo JC, Thompson D, Safdar N.** 2012. Is the Gram stain useful in the microbiologic diagnosis of VAP? A meta-analysis. Clin. Infect. Dis. **55:**551–561. http://dx.doi.org/10.1093/cid/cis512.
8. **Luna CM, Vujacich P, Niederman MS, Vay C, Gherardi C, Matera J, Jolly EC.** 1997. Impact of BAL data on the therapy and outcome of ventilator-associated pneumonia. Chest **111:**676–685. http://dx.doi.org/10.1378/chest.111.3.676.
9. **Pennington JE.** 1981. Penetration of antibiotics into respiratory secretions. Rev. Infect. Dis. **3:**67–73. http://dx.doi.org/10.1093/clinids/3.1.67.
10. **Limbago BM, Rasheed JK, Anderson KF, Zhu W, Kitchel B, Watz N, Munro S, Gans H, Banaei N, Kallen AJ.** 2011. IMP-producing carbapenem-resistant *Klebsiella pneumoniae* in the United States. J. Clin. Microbiol. **49:**4239–4245. http://dx.doi.org/10.1128/JCM.05297-11.
11. **Munoz-Price LS, Poirel L, Bonomo RA, Schwaber MJ, Daikos GL, Cormican M, Cornaglia G, Garau J, Gniadkowski M, Hayden MK, Kumarasamy K, Livermore DM, Maya JJ, Nordmann P, Patel JB, Paterson DL, Pitout J, Villegas MV, Wang H, Woodford N, Quinn JP.** 2013. Clinical epidemiology of the global expansion of *Klebsiella pneumoniae* carbapenemases. Lancet Infect. Dis. **13:**785–796. http://dx.doi.org/10.1016/S1473-3099(13)70190-7.
12. **Kollef MH.** 2005. What is ventilator-associated pneumonia and why is it important? Respir. Care **50:**714–721.
13. **Rosenfeld M, Cohen M, Ramsey B.** 1997. Aerosolized antibiotics for bacterial lower airway infections: principles, efficacy, and pitfalls. Clin. Pulm. Med. **4:**101–112. http://dx.doi.org/10.1097/00045413-199703000-00006.
14. **Rodríguez-Rojas A, Couce A, Blázquez J.** 2010. Frequency of spontaneous resistance to fosfomycin combined with different antibiotics in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **54:**4948–4949. http://dx.doi.org/10.1128/AAC.00415-10.
15. **Palmer LB, Smaldone GC, Chen JJ, Baram D, Duan T, Monteforte M, Varela M, Tempone AK, O'Riordan T, Daroowalla F, Richman P.** 2008. Aerosolized antibiotics and ventilator-associated tracheobronchitis in the intensive care unit. Crit. Care Med. **36:**2008–2013. http://dx.doi.org/10.1097/CCM.0b013e31817c0f9e.
16. **Lu Q, Yang J, Liu Z, Gutierrez C, Aymard G, Rouby JJ; Nebulized Antibiotics Study Group.** 2011. Nebulized ceftazidime and amikacin in ventilator-associated pneumonia caused by *Pseudomonas aeruginosa*. Am. J. Respir. Crit. Care Med. **184:**106–115. http://dx.doi.org/10.1164/rccm.201011-1894OC.
17. **Niederman MS, Chastre J, Corkery K, Fink JB, Luyt CE, Garcia MS.** 2012. BAY41-6551 achieves bactericidal tracheal aspirate amikacin concentrations in mechanically ventilated patients with Gram-negative pneumonia. Intensive Care Med. **38:**263–271. http://dx.doi.org/10.1007/s00134-011-2420-0.
18. **Montgomery AB, Vallance S, Abuan T, Tservistas M, Davies A.** 2 January 2014. A randomized double-blind placebo-controlled dose-escalation phase 1 study of aerosolized amikacin and fosfomycin delivered via the PARI Investigational eFlow® Inline Nebulizer System in mechanically ventilated patients. J. Aerosol Med. Pulm. Drug Deliv. http://dx.doi.org/10.1089/jamp.2013.1100.
19. **Montgomery AB, Rhomberg PR, Abuan T, Walters K-A, Flamm RK.** 2014. Potentiation effects of amikacin and fosfomycin against selected amikacin-nonsusceptible Gram-negative respiratory tract pathogens. Antimicrob. Agents Chemother. **58:**3714–3719. http://dx.doi.org/10.1128/AAC.02780-13.
20. **CLSI.** 2012. M07–A9. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard—9th ed. Clinical and Laboratory Standards Institute, Wayne, PA.
21. **CLSI.** 2013. M100–S23. Performance standards for antimicrobial susceptibility testing:23rd informational supplement. Clinical and Laboratory Standards Institute, Wayne, PA.
22. **Moody J, Knapp C.** 2010. Time-kill assay for determining synergy, p 5.10.3.1–5.10.3.6. *In* Garcia LS, Isenberg HD (ed), Clinical microbiology procedures handbook. ASM Press, Washington, DC.
23. **Moody J.** 2010. Synergism testing: broth microdilution checkerboard and broth macrodilution methods, p 5.12.1–5.12.23. *In* Garcia LS, Isenberg HD (ed), Clinical microbiology procedures handbook. ASM Press, Washington, DC.
24. **Sader HS, Huynh HK, Jones RN.** 2003. Contemporary in vitro synergy rates for aztreonam combined with newer fluoroquinolones and β-lactams tested against gram-negative bacilli. Diagn. Microbiol. Infect. Dis. **47:**547–550. http://dx.doi.org/10.1016/S0732-8893(03)00158-5.
25. **Kiffer CR, Sampaio JL, Sinto S, Oplustil CP, Koga PC, Arruda AC, Turner PJ, Mendes C.** 2005. In vitro synergy test of meropenem and sulbactam against clinical isolates of *Acinetobacter baumannii*. Diagn. Microbiol. Infect. Dis. **52:**317–322. http://dx.doi.org/10.1016/j.diagmicrobio.2005.03.003.
26. **Ramsey BW, Pepe MS, Quan JM, Otto KL, Montgomery AB, Williams-Warren J, Vasiljev KM, Borowitz D, Bowman CM, Marshall BC, Marshall S, Smith AL.** 1999. Intermittent administration of inhaled tobramycin in patients with cystic fibrosis. Cystic Fibrosis Inhaled Tobramycin Study Group. N. Engl. J. Med. **340:**23–30. http://dx.doi.org/10.1056/NEJM199901073400104.
27. **Oermann CM, McCoy KS, Retsch-Bogart GZ, Gibson RL, McKevitt M, Montgomery AB.** 2011. *Pseudomonas aeruginosa* antibiotic susceptibility during long-term use of aztreonam for inhalation solution (AZLI). J. Antimicrob. Chemother. **66:**2398–2404. http://dx.doi.org/10.1093/jac/dkr303.