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Synergistic Meropenem-Tobramycin Combination Dosage Regimens against Clinical Hypermutable *Pseudomonas aeruginosa* at Simulated Epithelial Lining Fluid Concentrations in a Dynamic Biofilm Model

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ABSTRACT Exacerbations of chronic *Pseudomonas aeruginosa* infections are a major treatment challenge in cystic fibrosis due to biofilm formation and hypermutation. We aimed to evaluate different dosage regimens of meropenem and tobramycin as monotherapies and in combination against hypermutable carbapenem-resistant *P. aeruginosa*. A hypermutable *P. aeruginosa* isolate (meropenem and tobramycin MICs, 8 mg/liter) was investigated in the dynamic CDC biofilm reactor over 120 h. Regimens were meropenem as the standard (2 g every 8 h, 30% epithelial lining fluid [ELF] penetration) and as a continuous infusion (CI; 6 g/day, 30% and 60% ELF penetration) and tobramycin at 10 mg/kg of body weight every 24 h (50% ELF penetration). The time courses of totally susceptible and less-susceptible bacteria and MICs were determined, and antibiotic concentrations were quantified by liquid chromatography-tandem mass spectrometry. All monotherapies failed, with the substantial regrowth of planktonic (>6 $\log_{10}$ CFU/ml) and biofilm (≥6 $\log_{10}$ CFU/cm²) bacteria occurring. Except for the meropenem CI (60% ELF penetration), all monotherapies amplified less-susceptible planktonic and biofilm bacteria by 120 h. The meropenem standard regimen with tobramycin caused initial killing followed by considerable regrowth with resistance (meropenem MIC, 64 mg/liter; tobramycin MIC, 32 mg/liter) for planktonic and biofilm bacteria. The combination containing the meropenem CI at both levels of ELF penetration synergistically suppressed the regrowth of total planktonic bacteria and the resistance of planktonic and biofilm bacteria. The combination with the meropenem CI at 60% ELF penetration, in addition, synergistically suppressed the regrowth of total biofilm bacteria. Standard regimens of meropenem and tobramycin were ineffective against planktonic and biofilm bacteria. The combination with meropenem CI exhibited enhanced bacterial killing and resistance suppression of carbapenem-resistant hypermutable *P. aeruginosa*.

KEYWORDS combination therapy, hypermutators, biofilm infections, antibiotic resistance, dosage regimens

Carbapenem-resistant *Pseudomonas aeruginosa* has been classified by the World Health Organization as one of the top three critical pathogens requiring new antibiotic treatments (1). *P. aeruginosa* has a particularly large armamentarium of resistance mechanisms and can develop resistance against virtually all antibiotics in
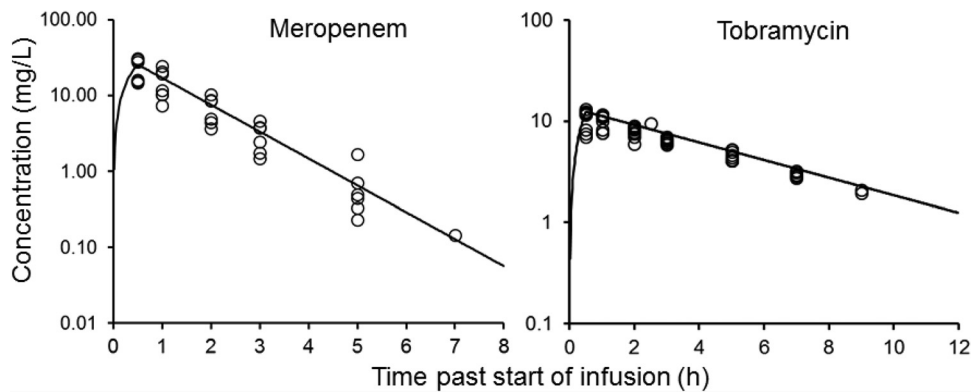
FIG 1 Pharmacokinetic profiles showing the relationship between the targeted (lines) and measured (symbols) meropenem and tobramycin concentrations in the CBR.

monotherapy. Such resistance development often results in treatment failure (2, 3). Respiratory infections caused by *P. aeruginosa* are a serious clinical problem for patients with cystic fibrosis (CF). Acute infective exacerbations (AIE) of chronic *P. aeruginosa* infections cause progressive lung function decline followed by respiratory failure (4, 5). Thus, they are a main driver of early death for patients with CF. Indeed, the rates of multidrug-resistance (MDR) for these infections in CF patients are substantially higher than those in patients in an intensive care unit (6).

The ability of *P. aeruginosa* to become hypermutable and to form a biofilm renders AIE especially difficult to treat (7, 8). Hypermutable isolates (i.e., those with an up to ~1,000-fold increased mutation rate due to defects in DNA repair or error avoidance systems) account for up to ~54% of *P. aeruginosa* strains in CF respiratory infections and are associated with reduced lung function (8–10). Despite an increased mutation rate, hypermutable strains generally do not show reduced fitness in the nutrient-rich environment of the CF lung. Their carriage is highly correlated with MDR, and hyper-mutation is important for biofilm development (8, 11). The formation of a biofilm hampers antibiotic effectiveness, e.g., via extracellular matrix formation, which reduces antibiotic penetration. Biofilm growth also leads to greater phenotypic diversity and, thus, a greater persistence of infections (12, 13). Therefore, biofilm-associated infections by hypermutable *P. aeruginosa* are extremely difficult to treat, especially when the hypermutable strains are MDR.

Current antibiotic regimens against *P. aeruginosa* infections in patients with CF are suboptimal; monotherapy is often ineffective, and combination regimens are used empirically (4, 14). We have demonstrated synergistic bacterial killing and the suppression of resistance emergence in hypermutable *P. aeruginosa* with a modified combination regimen of meropenem and tobramycin in the dynamic hollow-fiber infection model (HFIM) (15). However, treatment regimens against hypermutable *P. aeruginosa* have never been evaluated in a dynamic biofilm model, such as the Centers for Disease Control and Prevention biofilm reactor (CBR), that allows examination of antibacterial effects against both planktonic and biofilm bacteria. Thus, the aim of the present study was to use the CBR to simulate the concentration-time profiles for different meropenem and tobramycin dosage regimens observed in the epithelial lining fluid (ELF) of patients with CF when given as monotherapy and in combination and characterize the bacterial killing and resistance suppression of carbapenem-resistant hypermutable *P. aeruginosa*.

RESULTS

Pharmacokinetic validation, bacterial killing, and emergence of resistance. The pharmacokinetic profiles observed in the CBR (Fig. 1) were in good agreement with the targeted exposures (Table 1). The observed meropenem and tobramycin concentrations were, on average, within 10% of the targeted concentrations. The viable count profiles for planktonic and biofilm bacteria are presented in Fig. 2. The counts on

TABLE 1 Clinically representative ELF concentrations, exposures, and pharmacokinetic/pharmacodynamic indices for meropenem and/or tobramycin in the CBR[a]

Treatment	fC_{max}/fC_{min} or fC_{ss} (mg/liter)	$fAUC_{24}$ (mg·h/liter)	fC_{max}/MIC	$fT_{>MIC}$ (%)	$fAUC_{24}$/MIC
MER at 2 g every 8 h	25.4/0.06	115	3.18	22	
MER at 6 g/day as a CI	4.79	115	0	0	
MER at 6 g/day as a CI (60% ELF penetration)	9.58	230	1.20	100	
TOB at 10 mg/kg every 24 h	12.3/0.11	64.4	1.54		8.05

[a]MER, meropenem; TOB, tobramycin; CI, continuous infusion; fC_{max}, unbound maximum concentration; fC_{min}, unbound minimum concentration before the next dose; fC_{ss}, unbound average steady-state concentration; $fAUC_{24}$, the area under the unbound concentration-time curve over 24 h; fC_{max}/MIC, the ratio of the fC_{max} to the MIC; $fT_{>MIC}$, the cumulative percentage of a 24-h period that the unbound concentrations exceeded $1\times$ MIC; $fAUC_{24}$/MIC, the ratio of the $fAUC_{24}$ to the MIC. The simulated half-lives were 0.8 h for meropenem and 3.5 h for tobramycin. No loading dose was administered for intermittent dosing, whereas the modified meropenem dosage regimen (6-g/day CI) was started at the fC_{ss} of either 4.79 mg/liter (30% ELF penetration) or 9.58 mg/liter (60% ELF penetration). The simulated ELF penetration was 30% for meropenem, unless it is specified to be 60%, and was 50% for tobramycin.

antibiotic-containing agar are shown in Fig. 3, log changes in viable counts are shown in Table 2, mutation frequencies are shown in Table 3, and baseline and endpoint MICs are shown in Table 4.

Planktonic bacteria. The starting inoculum (mean ± standard error [SE]) in all arms was $7.19 \pm 0.05 \log_{10}$ CFU/ml ($n = 14$). *P. aeruginosa* CW8 in the control chambers grew to $7.75 \pm 0.15 \log_{10}$ CFU/ml by 24 h, and the growth plateaued at ~8.1 $\log_{10}$ CFU/ml (Fig. 2A). Colonies on drug-containing agar increased approximately in proportion to the growth of the total bacterial population (Fig. 3A, C, E, and G and Table 3).

Meropenem monotherapy simulating the standard regimen (2 g every 8 h) resulted in ~3-$\log_{10}$-CFU/ml initial killing at 3 h, followed by steady regrowth to ~7 $\log_{10}$ CFU/ml at 48 h, with slower regrowth toward control values occurring thereafter (Fig. 2A and Table 2). With this regimen, less-susceptible populations increased rapidly, with approximately half of the entire population growing on agar containing meropenem at 6 mg/liter by 120 h. Similar increases were observed on agar containing meropenem at 15 mg/liter, although growth remained ~1 log lower than the total population at 120 h. Emergence of resistance was observed, with an ~2.5-log increase of the meropenem-resistant bacteria compared to that for the growth control being seen at 120 h (Fig. 3A and C and Table 3). The MIC of colonies recovered from 15-mg/liter meropenem-containing plates at this time point was 128 mg/liter (Table 4). The bacterial killing achieved with the modified meropenem regimen at 6 g/day as a continuous infusion (CI) simulating 30% and 60% ELF penetration closely matched that achieved with the standard regimen (2 g every 8 h) over the first 24 h, but thereafter growth remained ~1 $\log_{10}$ CFU/ml (30% ELF penetration) and ~2 $\log_{10}$ CFU/ml (60% ELF penetration) lower than that for the standard regimen; for the treatment simulating 60% ELF penetration, regrowth at 120 h reached ~6 $\log_{10}$ CFU/ml (Fig. 2A and Table 2). While the increases in less-susceptible populations with the CI simulating 30% ELF penetration closely matched those for the standard regimen, the less-susceptible populations remained suppressed for 60% ELF penetration (Fig. 3A and C and Table 3). By 72 h with the latter treatment, growth on agar containing meropenem at 6 mg/liter and 15 mg/liter was ~2 and <1 $\log_{10}$ CFU/ml, respectively. Furthermore, the emergence of resistance was not observed at 120 h, and the MICs of colonies recovered from drug plates were increased by only 1 dilution (MIC, 16 mg/liter) (Table 4). Amplification of resistance with meropenem 30% ELF penetration was more evident, with an ~2.1-log increase in the meropenem-resistant population at 120 h in comparison to that for the growth control (Fig. 3C and Table 3). The MIC at this time point was 64 mg/liter (Table 4). The tobramycin monotherapy produced rapid (within the first 7 h) initial killing of ~3 $\log_{10}$ CFU/ml, followed by steady regrowth, such that growth approximated that of the growth control by 72 h (Fig. 2C and Table 2). Amplification of bacteria less susceptible to tobramycin was observed. The proportion of colonies growing on tobramycin-containing agar increased substantially over 120 h, with a large proportion of the entire population growing on plates containing 3 mg/liter tobramycin; on plates containing 7.5 mg/liter tobramycin, growth increased to within ~2
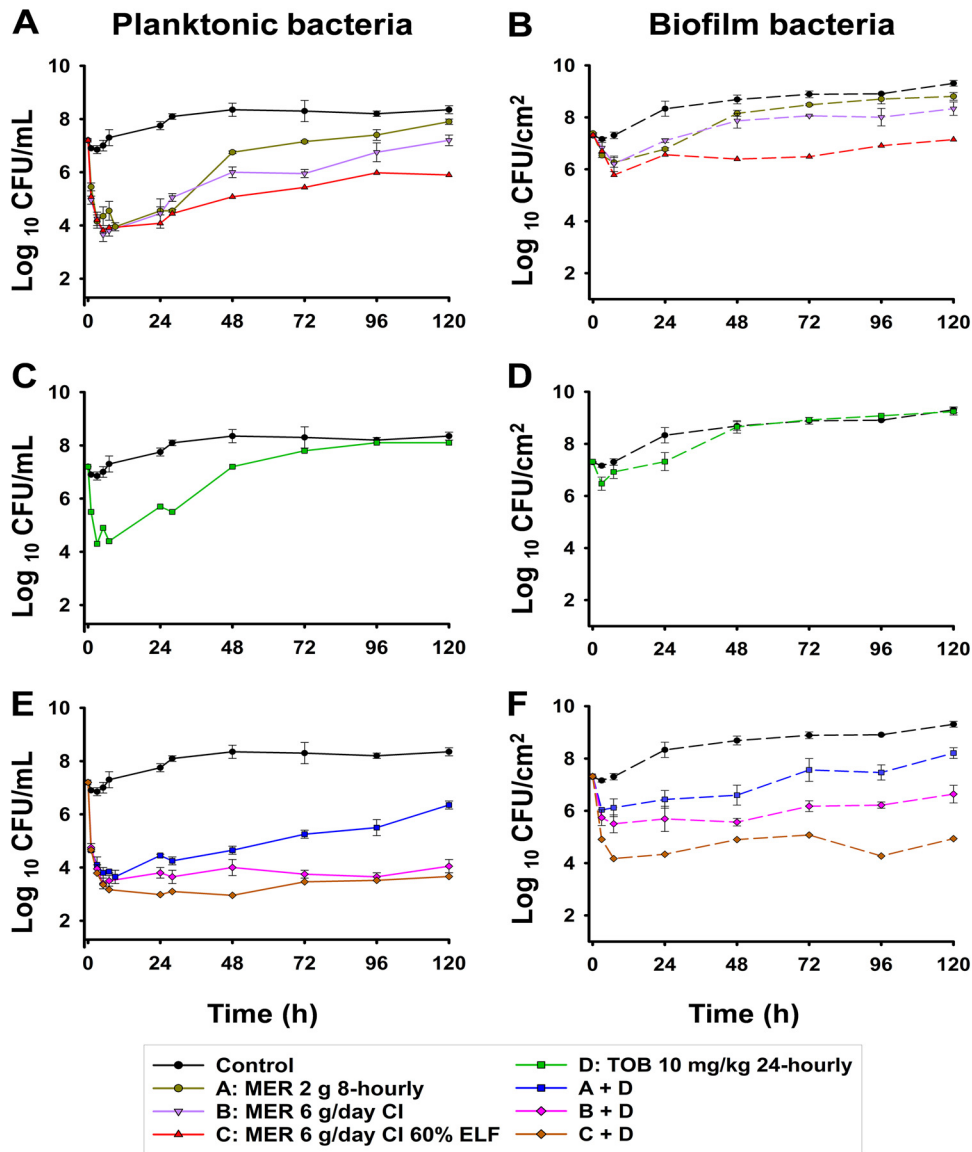
FIG 2 Total viable counts for the growth control and treatments with meropenem (MER) and/or tobramycin (TOB) at clinically relevant ELF concentration-time profiles sampled from the medium within the reactor, i.e., planktonic bacteria receiving meropenem monotherapy (A), tobramycin monotherapy (C), or the combination of meropenem and tobramycin (E), and from coupons, i.e., biofilm bacteria receiving meropenem monotherapy (B), tobramycin monotherapy (D), or the combination of meropenem and tobramycin (F). The results are presented as the average $\pm$ SE. The y axis starts from the limit of counting.

$\log_{10}$ CFU/ml of the total population (Fig. 3E and G and Table 3). The MIC at 120 h was 64 mg/liter (Table 4).

The combination containing the standard meropenem regimen produced $\sim$3.3-$\log_{10}$-CFU/ml initial killing at 5 h and regrowth to within $\sim$2 $\log_{10}$ CFU/ml of the growth of the growth control at 120 h (Fig. 2E and Table 2). The amplification of less-susceptible and resistant bacteria in comparison to that of the growth control was observed. Less-susceptible populations increased dramatically, such that virtually the entire population at 120 h grew on agar containing meropenem at 6 mg/liter and tobramycin at 3 mg/liter (Fig. 3A and E and Table 3). The MIC at this time point was 64 mg/liter for meropenem and 32 mg/liter for tobramycin (Table 4).

The combination treatment with the modified meropenem regimen with 30% ELF penetration produced initial killing of $\sim$3.8 $\log_{10}$ CFU/ml at 5 h, while that with 60% ELF penetration produced initial killing of $\sim$4.4 $\log_{10}$ CFU/ml at 24 h. Synergistic bacterial
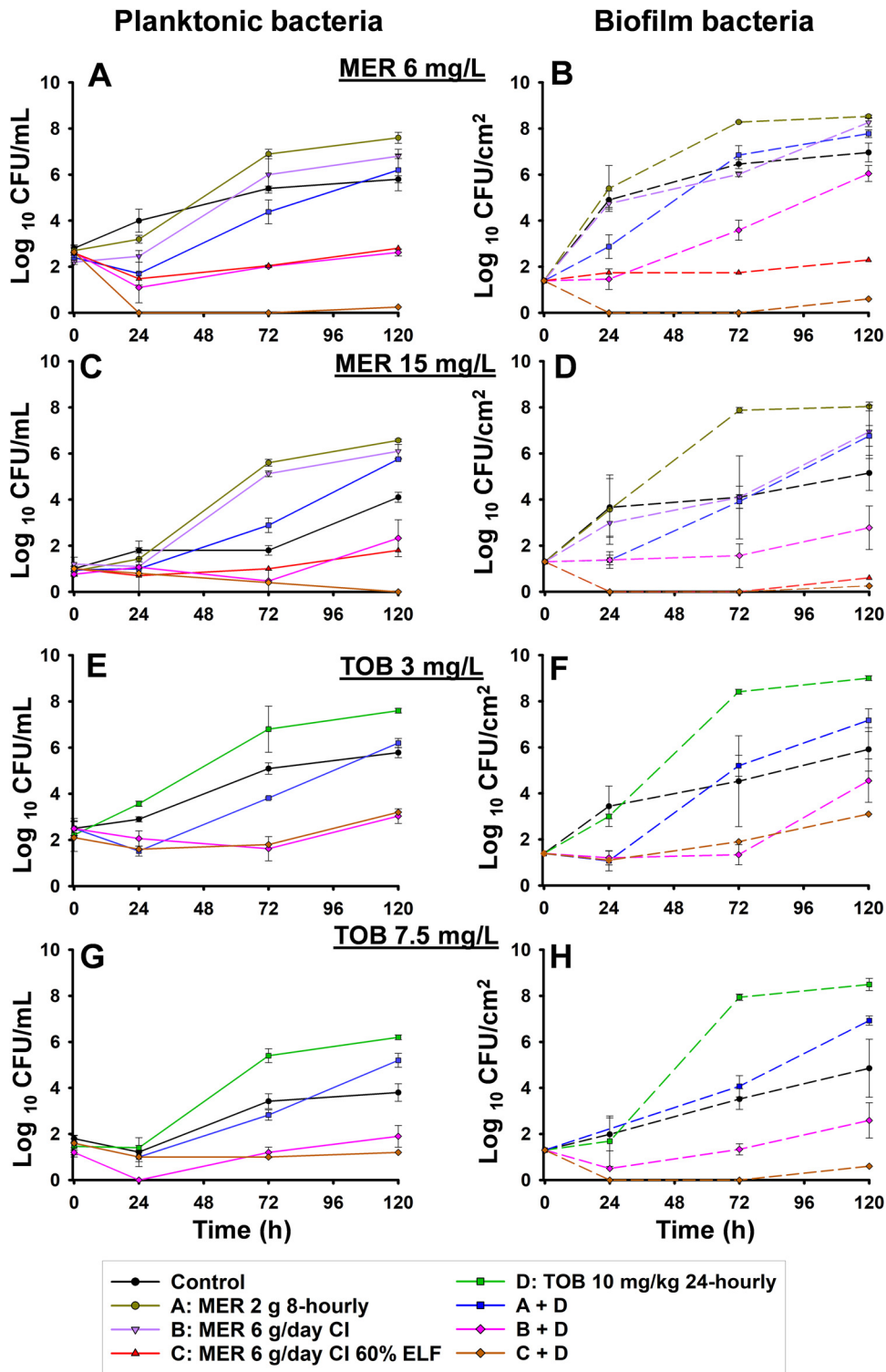
Planktonic bacteria

Biofilm bacteria

FIG 3 Effect of each dosage regimen on the counts of bacteria able to grow on agar plates containing 6 or 15 mg/liter of meropenem or 3 or 7.5 mg/liter of tobramycin. The results are represented as the average $\pm$ SE. To differentiate less-susceptible subpopulations from the predominant population, the antibiotic concentrations in agar were based upon Etest MICs, which were 1.5 mg/liter for meropenem and 0.75 mg/liter for tobramycin (9).

killing ($\geq 2 \log_{10}$ CFU/ml) was observed from 28 or 72 h onwards with both levels of ELF penetration; regrowth remained suppressed at $\sim$3.5 to 4 $\log_{10}$ CFU/ml at 120 h (Fig. 2E and Table 2). However, differences in the regrowth of less-susceptible populations were observed. On agar containing meropenem at 15 mg/liter, regrowth of $\sim$2 $\log_{10}$ CFU/ml

TABLE 2 Log changes in viable cell counts at various time points with clinically relevant ELF concentrations of meropenem and/or tobramycin[a]

Time (h)	A: MER 2g Q8h		B: MER 6g/d CI		C: MER 6g/d CI 60% ELF		D: TOB 10mg/kg Q24h		A + D		B + D		C + D	
	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria
1	-1.95		-2.25		-1.82		-1.70		-2.45		-2.45		-2.74	
3	-3.25	-0.85	-2.95	-0.47	-2.70	-0.62	-2.85	-0.43	-3.00	-1.28	-3.25	-1.34	-3.61	-2.41
5	-3.05		-3.55		-3.09		-2.70		-3.30		-3.80		-4.04	
7	-2.85	-1.02	-3.15	-1.58	-2.99	-0.74	-2.90	0.02	-3.25	-1.20	-3.70	-1.58	-4.23	-3.15
24	-2.85	-0.61	-2.75	-0.19	-2.82	-0.56	-1.80	0.41	-2.65	-0.88	-3.40	-1.69	-4.42	-2.98
28	-2.85		-2.15		-2.45		-1.35		-2.85		-3.55		-4.30	
48	-0.65	0.77	-1.20	0.58	-1.52	-0.92	-0.15	1.75	-2.45	-0.72	-3.20	-1.55	-4.45	-2.42
72	-0.25	1.10	-1.25	0.76	-1.47	-0.83	0.60	2.02	-1.85	0.25	-3.48	-0.90	-3.94	-2.25
96	0.10	1.32	-0.45	0.71	-0.92	-0.41	0.80	2.17	-1.60	0.15	-3.55	-0.86	-3.88	-3.05
120	0.60	1.42	-0.10	1.04	-1.00	-0.17	0.90	2.34	-0.75	0.89	-3.15	-0.44	-3.74	-2.38

[a]MER, meropenem; TOB, tobramycin; CFU_t, number of CFU at time t; CFU_0, number of CFU at time zero. The green background indicates synergy (a $\geq$2-$\log_{10}$ decrease in the number of CFU per milliliter or the number of CFU per square centimeter with the combination compared to the value for its most active component); the blue background indicates a 1.0- to <2-$\log_{10}$ decrease in the number of CFU per milliliter or the number of CFU per square centimeter with the combination compared to the value for its most active component.

was observed at 120 h with the combination simulating 30% ELF penetration of the meropenem CI (MIC, 32 mg/liter). However, virtually no colonies were detected from 24 h onwards at either meropenem plate concentration with the combination simulating 60% ELF penetration of meropenem. Growth on agar containing tobramycin at 7.5 mg/liter was ~1.5 to 2 $\log_{10}$ CFU/ml at 120 h for both combination treatments involving the meropenem CI, which was ~2 $\log_{10}$ CFU/ml below the growth control counts (Fig. 3G).

Biofilm-embedded bacteria. In the growth control, biofilm bacteria grew steadily to ~9 $\log_{10}$ CFU/cm² by 72 h and plateaued until 120 h (Fig. 2B); moderate increases

TABLE 3 $\log_{10}$ mutation frequencies at 6 mg/liter and 15 mg/liter meropenem and 3 mg/liter and 7.5 mg/liter tobramycin for each simulated regimen

		MEROPENEM				TOBRAMYCIN			
		6 mg/L		15 mg/L		3 mg/L		7.5 mg/L	
Time (h)	Arm	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria
0	All Regimens	-4.74	-5.93	-6.00	-6.02	-4.50	-5.93	-5.70	-5.82
24	Control	-3.87	-3.38	-5.77	-5.63	-4.97	-4.85	-5.60	-5.67
	A: MER 2g Q8h	-1.36	-1.38	-3.13	-3.21				
	B: MER 6g/d CI	-2.60	-2.36	-3.95	-4.12				
	C: MER 6g/d CI 60% ELF	-2.97	-4.16	-3.75	-6.76				
	D: TOB 10mg/kg Q24h					-1.98	-3.67	-4.15	-4.98
	A + D	-2.60	-3.57	-3.30	-5.06	-2.78	-4.37	-3.30	-5.44
	B + D	-2.24	-3.91	-2.27	-3.99	-1.27	-4.16	-3.33	-5.37
	C + D	-3.21	-4.34	-3.21	-4.34	-1.61	-3.24	-2.21	-4.34
72	Control	-2.80	-2.34	-5.40	-4.70	-3.11	-4.27	-4.78	-5.28
	A: MER 2g Q8h	-0.51	-0.20	-1.80	-0.89				
	B: MER 6g/d CI	-0.75	-2.04	-1.62	-3.96				
	C: MER 6g/d CI 60% ELF	-3.84	-4.75	-4.88	-6.49				
	D: TOB 10mg/kg Q24h					-1.30	-0.51	-2.70	-0.98
	A + D	-1.12	-0.72	-2.62	-3.55	-1.69	-2.36	-2.68	-3.49
	B + D	-1.39	-3.04	-2.93	-4.73	-1.78	-5.29	-2.20	-5.30
	C + D	-3.52	-5.07	-3.52	-5.07	-1.72	-3.17	-2.52	-5.07
120	Control	-2.57	-2.23	-4.27	-4.04	-2.58	-3.27	-4.57	-4.33
	A: MER 2g Q8h	-0.35	-0.28	-1.33	-0.61				
	B: MER 6g/d CI	-0.40	-0.07	-1.00	-1.39				
	C: MER 6g/d CI 60% ELF	-3.30	-4.86	-4.10	-6.55				
	D: TOB 10mg/kg Q24h					-0.45	-0.24	-1.85	-0.75
	A + D	-0.15	-0.38	-0.59	-1.34	-0.15	-1.03	-1.15	-1.28
	B + D	-1.11	-0.66	-1.40	-3.93	-0.70	-2.15	-2.58	-4.11
	C + D	-3.41	-4.33	-3.66	-4.68	-0.46	-1.83	-2.46	-4.33

[a]MER, meropenem; TOB, tobramycin. The red background indicates a high mutation frequency, i.e., a large proportion of less-susceptible bacteria being present in the total population; the green background indicates a low mutation frequency, i.e., a small proportion of less-susceptible bacteria being present in the total population.

TABLE 4 MIC values for colonies obtained from antibiotic-containing agar plates at 0 and 120 h for each dosage regimen[a]

| Treatment | Meropenem at 15 mg/liter | | | Tobramycin at 7.5 mg/liter | | |
| | Time (h) | MIC (mg/liter) | | Time (h) | MIC (mg/liter) | |
		Planktonic bacteria	Biofilm bacteria		Planktonic bacteria	Biofilm bacteria
Control	0	16	8	0	16	8
	120	32	16	120	32	16
MER at 2 g every 8 h	120	128	32	—	—	—
MER at 6 g/day as a CI	120	64	32	—	—	—
MER at 6 g/day as a CI (60% ELF penetration)	120	16	NC	—	—	—
TOB at 10 mg/kg every 24 h	—	—	—	120	64	32
MER at 2 g every 8 h + TOB at 10 mg/kg every 24 h	120	64	32	120	32	32
MER at 6 g/day as a CI + TOB at 10 mg/kg every 24 h	120	32	16	120	32	16
MER at 6 g/day as a CI (60% ELF penetration) + TOB at 10 mg/kg every 24 h	120	NC	NC	120	NC	NC

[a]The agar plates contained meropenem (MER) at 15 mg/liter and tobramycin (TOB) at 7.5 mg/liter. NC, no colonies grew on the antibiotic-containing plates; —, not tested.

in the proportion of less-susceptible populations were observed across 120 h (Fig. 3B, D, F, and H and Table 3). Following 7 h of treatment, bacterial killing from all monotherapy regimens was ~1 $\log_{10}$ CFU/cm². After that, regrowth occurred with all regimens except the meropenem CI with 60% ELF penetration, such that growth was within ≤2 $\log_{10}$ CFU/cm² of that of the control from 48 h onwards; with the CI (60% ELF penetration), growth remained at ~6.5 to 7.0 $\log_{10}$ CFU/cm² across 120 h (Fig. 2B and D and Table 2). Substantial increases in less-susceptible populations occurred with the tobramycin and both the standard and CI (30% ELF penetration) meropenem regimens but not with the CI (60% ELF penetration) meropenem regimen (Fig. 3B, D, F, and H and Table 3). With the latter regimen, growth on agar containing meropenem at 6 mg/liter remained steady at ~2 $\log_{10}$ CFU/cm² across 120 h, whereas virtually no colonies were detected on agar containing meropenem at 15 mg/liter from 24 h onwards. At 120 h, the MIC was 32 mg/liter for the meropenem standard regimen and the CI at 30% ELF penetration; the MIC for the tobramycin regimen was also 32 mg/liter (Table 4).

The combination containing the standard meropenem regimen simulating 30% ELF penetration produced ~1-$\log_{10}$-CFU/cm² initial killing at 3 h, followed by regrowth to within ~1 $\log_{10}$ CFU/cm² of the growth of the growth control at 120 h (Fig. 2F and Table 2). Amplification of bacteria less susceptible to meropenem and tobramycin in comparison to that of the growth control was observed (Fig. 3B, D, F, and H and Table 3). The MIC for both meropenem and tobramycin was 32 mg/liter at 120 h (Table 4). In contrast, with the combination regimens simulating the meropenem CI at 30% or 60% ELF penetration, a more substantial antibacterial effect was observed. With the meropenem CI at 30% ELF penetration, biofilm bacteria remained suppressed below ~6 $\log_{10}$ CFU/cm² up to 48 h, with only ~1 $\log_{10}$ CFU/cm² of growth occurring thereafter (Fig. 2F and Table 2). At 24 and 48 h, bacterial counts were 1 to 2 $\log_{10}$ CFU/cm² lower for the combination than for the most active monotherapy. Bacteria less susceptible to meropenem and tobramycin grew to within ~2 $\log_{10}$ CFU/cm² of the growth of the growth control by 120 h (Fig. 3B, D, F, and H). The MIC for meropenem and tobramycin was 16 mg/liter at this time point (Table 4). The combination simulating the meropenem CI at 60% ELF penetration achieved ~2.3- to 3.2-$\log_{10}$-CFU/cm² bacterial killing from 7 h onwards (Fig. 2F and Table 2); synergistic bacterial killing (≥2 $\log_{10}$ CFU/cm² compared to the killing obtained with the most active monotherapy) was observed at 24, 96, and 120 h (Fig. 2B and F and Table 2). A negligible number (~0.5 $\log_{10}$ CFU/cm²) of colonies was observed on plates containing tobramycin at 7.5 mg/liter or meropenem at 6 or 15 mg/liter (Fig. 3B, D, F, and H).

DISCUSSION

This study systematically investigated the bacterial killing and resistance suppression of standard versus modified dosage regimens of meropenem and tobramycin

against a carbapenem-resistant clinical hypermutable *P. aeruginosa* isolate in the CBR. The pharmacokinetic profiles simulated were representative of the unbound antibiotic concentrations expected in the ELF of patients with CF.

Pharmacokinetic/pharmacodynamic approaches to optimize the administration of β-lactams, including meropenem, traditionally involve maximizing the cumulative percentage of a 24-h period that the unbound concentrations exceed 1× MIC ($fT_{>MIC}$) for the infecting pathogen (16, 17). For serious bacterial infections, targets such as 100% $fT_{>4-5×MIC}$ (18–20) have been proposed. The $fT_{>MIC}$ can be modulated by altering the mode of administration. In the present investigations, meropenem was delivered either as a short-term infusion (standard regimen) or as a continuous infusion (CI; modified regimen), representing the extreme modes of administration in clinical practice. Importantly, however, the above-mentioned targets relate to planktonic bacteria; targets for biofilm bacteria are yet to be established and likely to be higher.

In our CBR studies, all meropenem regimens in monotherapy, at both levels of ELF penetration, were unable to suppress regrowth to below ~6 $\log_{10}$ for both planktonic and biofilm bacteria (Fig. 2). Even the CI with 60% ELF penetration was not successful, despite achieving a 100% $fT_{>MIC}$. This comprehensive failure of meropenem regimens strongly argues against the use of meropenem as monotherapy against a meropenem-resistant hypermutable *P. aeruginosa* isolate. For a meropenem-susceptible hypermutable *P. aeruginosa* strain, we have previously demonstrated in a 10-day HFIM study that the CI of meropenem to achieve a concentration as high as ~8× MIC was unable to suppress the emergence of less-susceptible planktonic bacteria (15). In the current study, biofilm bacteria were more resilient to meropenem than planktonic bacteria (Fig. 2). The bacterial cells in a biofilm are difficult to kill because of multiple factors. This includes the low metabolic activity of subpopulations located in the inner parts of the biofilm; e.g., low peptidoglycan production affects bacterial killing by meropenem (21). In a recent study of *P. aeruginosa* in a biofilm, meropenem concentrations substantially higher than those expected in ELF achieved some activity against meropenem-susceptible strains, while there was no activity against a meropenem-resistant strain (22).

For aminoglycosides, such as tobramycin, the ratio of exposure across a 24-h period to the MIC (the area under the unbound concentration-time curve [$fAUC$]/MIC) and the ratio of the unbound maximum concentration (fC_{max}) to the MIC (fC_{max}/MIC) have been correlated with antibacterial activity. An $fAUC$/MIC of >70 and an fC_{max}/MIC of 8 to 10 have been proposed as clinical targets (23); in the present study, the corresponding values were ~8 and ~1.5 (Table 1). For cationic antimicrobials, such as tobramycin, the presence of extracellular DNA in the biofilm decreases activity via chelation (24, 25). Thus, it is not surprising that the tobramycin regimen was ineffective in suppressing regrowth, resulting in a large increase of less-susceptible planktonic and biofilm bacteria (Fig. 2 and 3). This result was in agreement with that of our previous *in vitro* study, where tobramycin monotherapy failed to inhibit the regrowth of planktonic hypermutable *P. aeruginosa* even for $fAUC$/MIC values of 72 and 168 (26).

In the CBR, the combination containing the standard meropenem regimen (30% ELF penetration) with tobramycin resulted in a significant regrowth of less-susceptible planktonic and biofilm bacteria. Total bacterial counts were within 1 log of those achieved with the most active monotherapy at each time point. Importantly, the combination containing the modified meropenem (CI) regimen achieved enhanced bacterial killing and resistance suppression. This combination suppressed the regrowth and resistance of planktonic bacteria over 5 days at both levels of ELF penetration, demonstrating clear synergy. Although some regrowth of total biofilm bacteria was observed from 48 h onwards when simulating 30% ELF penetration of meropenem, resistant subpopulations remained suppressed compared to the growth control over 5 days. It is not surprising that the combination with standard meropenem dosing was less effective than that with the meropenem CI, as in the former case there were substantial periods with essentially no antibiotic present for activity. The combination simulating 60% ELF penetration synergistically suppressed the regrowth of both total

and resistant biofilm bacteria over 5 days. The synergy observed was notable, given that the isolate was meropenem resistant and tobramycin intermediate.

The synergistic bacterial killing and suppression of resistance observed in our study may be due to the different mechanisms of action and resistance of each antibiotic. Meropenem inhibits cell wall synthesis via binding to penicillin-binding proteins (27), and the main mechanisms of resistance in *P. aeruginosa* involve AmpC β-lactamase overexpression, reduced outer membrane porin OprD, and enzymatic inactivation via carbapenemases (28). Tobramycin predominantly acts by protein synthesis inhibition (29) but also by the disruption of the outer bacterial membrane (30–32). Resistance mechanisms against tobramycin include target site modification, enzymatic cleavage, increased expression of MexXY-OprM, and reduced outer membrane permeability (33, 34). For CW8, we previously identified mutations in genes related to both meropenem (*oprD*, *ampC*, *ampR*) and tobramycin (*fusA1*) resistance (9). Although the effects of each of the antibiotics in the combination were attenuated due to resistance, our results indicate that exposing the bacteria to both antibiotics simultaneously had a beneficial effect. In addition, mechanistic synergy may have been caused by tobramycin enhancing the target site penetration of meropenem (15). We have demonstrated that tobramycin in combination with another carbapenem, imipenem, caused extensive ultrastructural disruption of the outer membrane (32). This mechanistic synergy may apply not only to planktonic bacteria but also to biofilm bacteria.

To the best of our knowledge, this is the first study to examine the activity of the meropenem and tobramycin combination against carbapenem-resistant hypermutable *P. aeruginosa* in planktonic and biofilm growth by simulating ELF pharmacokinetics. Two studies in the dynamic HFIM (15, 18) and one *in vivo* infection model study (35) also examined the activity of this combination against *P. aeruginosa*. It is important to note that these studies involved susceptible isolates and investigated only planktonic growth, and the *in vitro* studies represented plasma rather than ELF concentrations. Two static concentration time-kill studies previously examined the activity of the same combination at a range of concentrations against susceptible planktonic *P. aeruginosa* (36, 37). The effect of the meropenem-tobramycin combination on the biofilm biomass of a susceptible *P. aeruginosa* strain has also been studied using a dynamic flow cell model and microscopy (38). However, that study did not quantify the counts of either biofilm or planktonic bacteria, nor did it examine the emergence of resistance, and the concentrations were higher than those achievable in ELF after intravenous dosing.

The current study has a number of strengths. It is the first study to examine the activity of the meropenem-tobramycin combination against a carbapenem-resistant hypermutable *P. aeruginosa* isolate. Furthermore, this is the only dynamic *in vitro* study to utilize concentration-time profiles representative of those in ELF for this combination. Since different levels of ELF penetration of meropenem have been reported (39–41), we examined both 30% and 60% ELF penetration. Importantly, the combination with the modified meropenem regimen achieved enhanced bacterial killing and resistance suppression even at the low ELF penetration. This study was conducted over 5 days of treatment, quantified both biofilm and planktonic bacteria, and evaluated the emergence of resistance. In addition, multiple biological replicates were performed to demonstrate the reproducibility of the total and less-susceptible bacterial counts. It is also important to note some limitations. The comprehensive studies conducted involved one clinical *P. aeruginosa* isolate. Future studies may be directed at evaluating the effect of the combination against other isolates, investigating its effect on the biofilm structure via confocal microscopy, and developing a mechanism-based mathematical model for antibiotic effects on biofilm bacteria. In addition, as with all other *in vitro* models, the CBR lacks an immune system. Therefore, future animal studies may be warranted to assess immune system effects on residual populations following the initial bacterial killing by the antibiotics. However, the accurate representation of humanized pharmacokinetic profiles is challenging in animal models due to the differences in clearance and half-life. In addition, given the ethical limitations on study duration inherent in animal studies, suppression of the emergence of resistance, a key compo-

nent of the present study, is best examined in the CBR, where longer study durations can be employed.

In conclusion, standard regimens of meropenem and tobramycin, both as monotherapy and in combination, were ineffective in suppressing regrowth and the emergence of resistance in both planktonic and biofilm bacteria. Importantly, however, the combination with the meropenem continuous infusion regimen, at both levels of ELF penetration, exhibited enhanced bacterial killing and resistance suppression against carbapenem-resistant hypermutable *P. aeruginosa*. Thus, this promising combination regimen warrants further evaluation.

MATERIALS AND METHODS

Bacterial isolate, antibiotics, and MICs. A previously described clinical hypermutable *P. aeruginosa* isolate (CW8) was employed (9). Hypermutability was defined as a mutation frequency on rifampin-containing agar at least 20-fold higher than that obtained for the control strain, PAO1 (9, 10). Sterile stock solutions of meropenem (lot Maus1025; Kabi, Melbourne, Australia) and tobramycin (lot LC24138; AK Scientific, Union City, MD, USA) were prepared in Milli-Q water immediately prior to each experiment. The MICs determined in duplicate on separate days using agar dilution per Clinical and Laboratory Standards Institute (CLSI) guidelines were 8 mg/liter for each antibiotic (42). Susceptibility and resistance were defined as MICs of ≤2 mg/liter and ≥8 mg/liter, respectively, for meropenem, and ≤4 mg/liter and ≥16 mg/liter, respectively, for tobramycin, per CLSI guidelines (42). The isolate was resistant to meropenem, intermediate to tobramycin, and MDR, based on agar dilution MICs. MDR was defined as nonsusceptibility to at least one antimicrobial agent in three or more antimicrobial categories (43). The biofilm formation capacity of CW8 was confirmed by the crystal violet assay.

In vitro **dynamic biofilm model, quantification of bacterial killing, emergence of resistance, and dosage regimens.** The time courses of bacterial killing and the emergence of resistance of planktonic and biofilm-embedded bacteria for the standard and modified regimens of meropenem and tobramycin as monotherapy and in combination were investigated over 120 h using the CBR (Bio Surface Technologies, Bozeman, MT, USA). The CBR model consisted of a 1-liter glass reactor connected to a 10-liter carboy containing sterile drug-free cation-adjusted Mueller-Hinton broth (CAMHB; BD, Sparks, MD, USA) containing 25 mg/liter Ca^{2+}, 12.5 mg/liter Mg^{2+}, and 1% tryptic soy broth (TSB) (CAMHB–1% TSB). Broth was pumped through the model (broth volume in the reactor, 350 ml), along with mixing, and shear was generated by a magnetic stir bar operating at 130 rpm. A hot plate maintained the CAMHB–1% TSB at 35°C. The biofilm formed on removable polycarbonate coupons (diameter, 12.7 mm) located in eight polypropylene coupon holders suspended from the reactor lid (three coupons per holder); the total surface area of each coupon was 2.53 cm².

The protocol for biofilm growth was similar to that described in our previous study (44). Prior to each experiment, CW8 was subcultured onto cation-adjusted Mueller-Hinton agar (CAMHA) containing 25 mg/liter Ca^{2+} and 12.5 mg/liter Mg^{2+} (Media Preparation Unit, University of Melbourne, Melbourne, Australia) and incubated at 35°C for 48 h. Following incubation, 2 or 3 random colonies were selected and grown overnight in 10 ml TSB, from which early-log-phase growth was obtained. A 28-h conditioning phase was then commenced via inoculation of 1 ml of this suspension into the model. Conditioning initially involved 24 h of incubation in drug-free CAMHB–1% TSB. Subsequently, all CAMHB–1% TSB was removed (to expel all planktonic bacteria and allow the amplification of bacteria shedding from the biofilm) and the reactor was refilled with drug-free CAMHB–1% TSB, pumped through the model for 4 h (flow rate, 11.67 ml/min) prior to the commencement of antibiotic treatment (i.e., 0 h) (44). The presence of biofilm on the coupons was confirmed by electron microscopy at 0 h.

At 0 h, the flow rate was changed to 4.9 ml/min for all treatments, to simulate a meropenem ELF elimination half-life ($t_{1/2}$) of ~0.8 h, reflecting that in patients with CF (45). For tobramycin-containing treatments, tobramycin was supplemented to achieve the required ELF $t_{1/2}$ of ~3.5 h (46, 47). For intermittent infusions, the antibiotics were administered using syringe drivers. The meropenem CI was achieved by administering a loading dose at 0 h directly into the reactor to immediately attain the required steady-state ELF concentration and spiking the meropenem stock solution into the carboy to maintain the steady-state concentration. Meropenem and tobramycin have negligible plasma protein binding; therefore, the concentrations used represent unbound (free) concentrations (Table 1). For meropenem, two regimens utilizing the highest FDA-recommended daily dose (6 g/day) were selected: the standard regimen of 2 g three times daily via a 30-min intravenous infusion with an fC_{max} of 25.3 mg/liter (Table 1) and a modified regimen of 6 g/day as a continuous infusion (CI) with a loading dose to rapidly achieve the unbound average steady-state concentration(fC_{ss}; Table 1). The modified regimen was simulated for both 30% and 60% ELF penetration. The pharmacokinetic profiles simulated in the CBR were based on the antibiotic concentrations over time that would be expected in the ELF of CF patients given the respective regimens. These expected unbound antibiotic concentration-time profiles were simulated *in silico* using the Berkeley Madonna (version 8.3.18) program (48), based on clinical studies and population pharmacokinetic models for CF patients (45, 46). The rate and extent of penetration of meropenem (30%) and tobramycin (50%) into ELF were derived from multiple published studies in patients (39, 41, 47). For meropenem, a higher ELF penetration (60%) based on a healthy volunteer study (40) was also considered. For tobramycin, the highest FDA-recommended daily dose for CF patients (10 mg/kg of body weight) was administered as a 30-min intravenous infusion every 24 h to yield the area under the unbound concentration-time curve over 24 h ($fAUC_{24}$) of 64.4 mg·h/liter

(Table 1). A growth control was also included. With one exception, all control and drug-containing regimens were performed in two replicates. Syringe drivers were tested and flow rates through the CBR were calibrated prior to each study and monitored throughout to ensure that the system was performing optimally.

Samples for viable counting were collected at 0, 1, 2, 3, 5, 7, 24, 28, 48, 72, 96, and 120 h for planktonic bacteria (1 ml) and at 0, 3, 7, 24, 48, 72, 96, and 120 h for biofilm-embedded bacteria. For biofilm bacteria, a coupon holder containing three coupons was aseptically replaced with a blank holder at each time point. The removed coupons were rinsed twice in 10 ml of phosphate-buffered saline (PBS; pH 7.4) to remove planktonic cells and then placed in sterile tubes containing 10 ml PBS. Three alternating 1-min cycles of vortexing and sonication at 43 kHz followed by a final 1 min of vortexing were used to extract the biofilm-embedded cells (44). For the enumeration of the total bacterial population, 100 μl of appropriately diluted sample was manually plated onto drug-free CAMHA and incubated at 35°C for 48 h, due to the slow growth of the hypermutable CW8 isolate. The number of bacteria recovered from the coupons was expressed as the number of $\log_{10}$ CFU per square centimeter. Less-susceptible subpopulations were quantified for planktonic and biofilm bacteria at 0 (pretreatment), 24, 72, and 120 h following the start of treatment by plating 200 μl of appropriately diluted sample onto CAMHA (BD, Sparks, MD, USA) supplemented with meropenem at 6 mg/liter or 15 mg/liter or tobramycin at 3 mg/liter or 7.5 mg/liter. The plates were incubated for 48 h (meropenem) or 72 h (tobramycin) (15). MICs were determined at 0 and 120 h by the agar dilution method for colonies isolated from antibiotic-containing plates.

Pharmacokinetic validation. For antibiotic-containing regimens, 1-ml samples were collected in duplicate from the CBR at multiple time points across the duration of the study and immediately stored at −80°C. Meropenem and tobramycin in CAMHB–1% TSB were measured using validated liquid chromatography-tandem mass spectrometry assays (15). The protocol for measuring tobramycin and meropenem was similar to that described in our previous study (15), except for slight modifications required by the presence of TSB. Modifications included the gradient of the binary mobile phase, programmed as 0.25% formic acid (A)–acetonitrile (B) at 80:20 that changed over 0.5 min to A-B at 50:50, which was held for 2.51 min, followed by reequilibration to A-B at 80:20 for 4.59 min. The flow rate of the mobile phase was 0.3 ml/min, the column oven temperature was 30°C, and the total run time was 7 min. The lower limit of quantification was 0.10 mg/liter for meropenem and 0.50 mg/liter for tobramycin. The correlation coefficients for the calibration curve of meropenem (range, 0.10 to 50.0 mg/liter) and tobramycin (range, 0.50 to 25.0 mg/liter) were >0.998 and >0.999, respectively. The interday precisions were 1.1 to 5.8% for meropenem and 2.1 to 7.5% for tobramycin; interday accuracies were 96.3 to 106.9% for meropenem and 95.9 to 102.1% for tobramycin.

Pharmacodynamic analysis. Monotherapy or combination regimens causing a reduction of ≥1 $\log_{10}$ CFU/ml or CFU/cm² at a specified time relative to the baseline were considered active. Synergy was defined as ≥2 $\log_{10}$-CFU/ml or -CFU/cm² killing for the combination relative to that for the most active corresponding monotherapy at a specified time. Bacterial counts on antibiotic-containing plates were used to evaluate resistance emergence for different treatment regimens in comparison to the growth control. Mutation frequencies were calculated as the difference between the number of $\log_{10}$ CFU per milliliter (number of $\log_{10}$ CFU per square centimeter) on antibiotic-containing agar and the number of $\log_{10}$ CFU per milliliter (number of $\log_{10}$ CFU per square centimeter) on antibiotic-free agar at the same time point.

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