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PHARMACOLOGY

Polymyxin B in Combination with Rifampin and Meropenem against Polymyxin B-Resistant KPC-Producing *Klebsiella pneumoniae*

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ABSTRACT Safe and effective therapies are urgently needed to treat polymyxin-resistant KPC-producing *Klebsiella pneumoniae* infections and suppress the emergence of resistance. We investigated the pharmacodynamics of polymyxin B, rifampin, and meropenem alone and as polymyxin B-based double and triple combinations against KPC-producing *K. pneumoniae* isolates. The rates and extents of killing with polymyxin B (1 to 128 mg/liter), rifampin (2 to 16 mg/liter), and meropenem (10 to 120 mg/liter) were evaluated against polymyxin B-susceptible (PB^s) and polymyxin B-resistant (PB^r) clinical isolates using 48-h static time-kill studies. Additionally, humanized triple-drug regimens of polymyxin B (concentration at steady state [C_{ss}] values of 0.5, 1, and 2 mg/liter), 600 mg rifampin every 12 or 8 h, and 1 or 2 g meropenem every 8 h dosed as an extended 3-h infusion were simulated over 48 h by using a one-compartment *in vitro* dynamic infection model. Serial bacterial counts were performed to quantify the pharmacodynamic effect. Population analysis profiles (PAPs) were used to assess the emergence of polymyxin B resistance. Monotherapy was ineffective against both isolates. Polymyxin B with rifampin demonstrated early bactericidal activity against the PB^s isolate, followed by regrowth by 48 h. Bactericidal activity was sustained at all polymyxin B concentrations of ≥2 mg/liter in combination with meropenem. No two-drug combinations were effective against the PB^r isolate, but all simulated triple-drug regimens showed early bactericidal activity against both strains by 8 h that was sustained over 48 h. PAPs did not reveal the emergence of resistant subpopulations. The triple-drug combination of polymyxin B, rifampin, and meropenem may be a viable consideration for the treatment of PB^r KPC-producing *K. pneumoniae* infections. Further investigation is warranted to optimize triple-combination therapy.

KEYWORDS polymyxin B, carbapenemase, *Klebsiella pneumoniae*, triple combination, pharmacodynamics

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The global escalation of antimicrobial resistance can be attributed in part to bacterial production of β-lactamases, which hydrolyze β-lactam antibiotics and ultimately render them inactive. The production of *Klebsiella pneumoniae* carbapenemase (KPC) enzymes by *K. pneumoniae* confers broad-spectrum resistance to most β-lactam agents, including carbapenems. Furthermore, these enzymes, expressed by genes on transferable plasmids, have led to widespread resistance throughout the *Enterobacteriaceae* family (1–4). The prevalence of infections due to *Klebsiella* spp. resistant to nearly all currently available antibiotics has increased over the past decade, and these infections

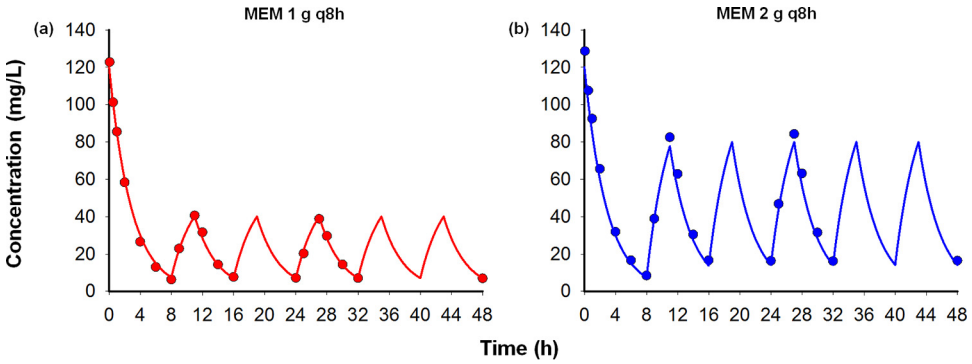

FIG 1 Pharmacokinetic profiles of extended-interval dosing of meropenem at 1 g (a) and 2 g (b) in one-compartment models. Solid lines indicate targeted meropenem concentration-time profiles, and closed circles indicate mean observed concentrations. MEM, meropenem; q8h, every 8 h.

are responsible for over 87% of carbapenem-resistant infections per year (5). Mortality rates of >40% have been reported for patients with KPC-producing *K. pneumoniae* infections (6–8). Hence, the Centers for Disease and Control Prevention (CDC) categorized carbapenem-resistant *Enterobacteriaceae* as an "urgent threat."

The polymyxins (polymyxin B and colistin [polymyxin E]) are utilized as last-line therapy against KPC producers (9, 10). However, the increased use of polymyxins has led to resistance, likely arising from suboptimal dosing coupled with the presence of heteroresistance (11, 12). KPC-producing *K. pneumoniae* strains harboring polymyxin resistance mechanisms have been reported globally (13–16). This poses a serious public health problem, as infections due to polymyxin-resistant strains are an independent predictor of mortality (17–19). Given the shortage of novel agents in the drug pipeline and an absence of clinical studies to evaluate polymyxin dosing strategies, optimization of existing antibiotics and their combinations is a useful preclinical step for evaluating novel, safe, and effective treatment options to combat polymyxin-resistant KPC-producing *K. pneumoniae* and suppress the emergence of resistance.

Previous *in vitro* studies demonstrated synergy between two-drug combinations of polymyxin and rifampin or meropenem against KPC-producing *K. pneumoniae* (20–24). Thus, the objective of our study was to evaluate the pharmacodynamic (PD) activities of polymyxin B, rifampin, and meropenem alone and as polymyxin B-based double and triple combinations against polymyxin B-sensitive (PB^s) and polymyxin B-resistant (PB^r) KPC-producing *K. pneumoniae* isolates using time-kill studies. Furthermore, using a funneling approach, we simulated clinically relevant dosing regimens in a dynamic *in vitro* one-compartment model to further evaluate the effect of triple therapy on the rate and extent of killing and the emergence of polymyxin resistance in these *K. pneumoniae* isolates.

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RESULTS

Pharmacokinetic (PK) validation. The polymyxin B concentrations at steady state (C_{ss}) (means ± standard deviations) were 0.46 ± 0.07 mg/liter (n = 8), 1.08 ± 0.11 mg/liter (n = 8), and 1.95 ± 0.11 mg/liter (n = 8) for targets of 0.5, 1, and 2 mg/liter, respectively. The rifampin maximum concentration (C_{max}) at steady state was 4.67 ± 0.24 mg/liter (n = 24). The observed and targeted mean unbound concentration-time profiles for extended-interval dosing of 1 g and 2 g meropenem used for *in vitro* dynamic infection model (IVDIM) studies are shown in Fig. 1. The close agreement between the observed and target concentrations indicates that the appropriate concentration-time profiles were achieved over 48 h.

Pharmacodynamic activity. (i) Monotherapy. Time-kill curves for the polymyxin B, rifampin, and meropenem concentrations evaluated against PBS_BAA1705 and

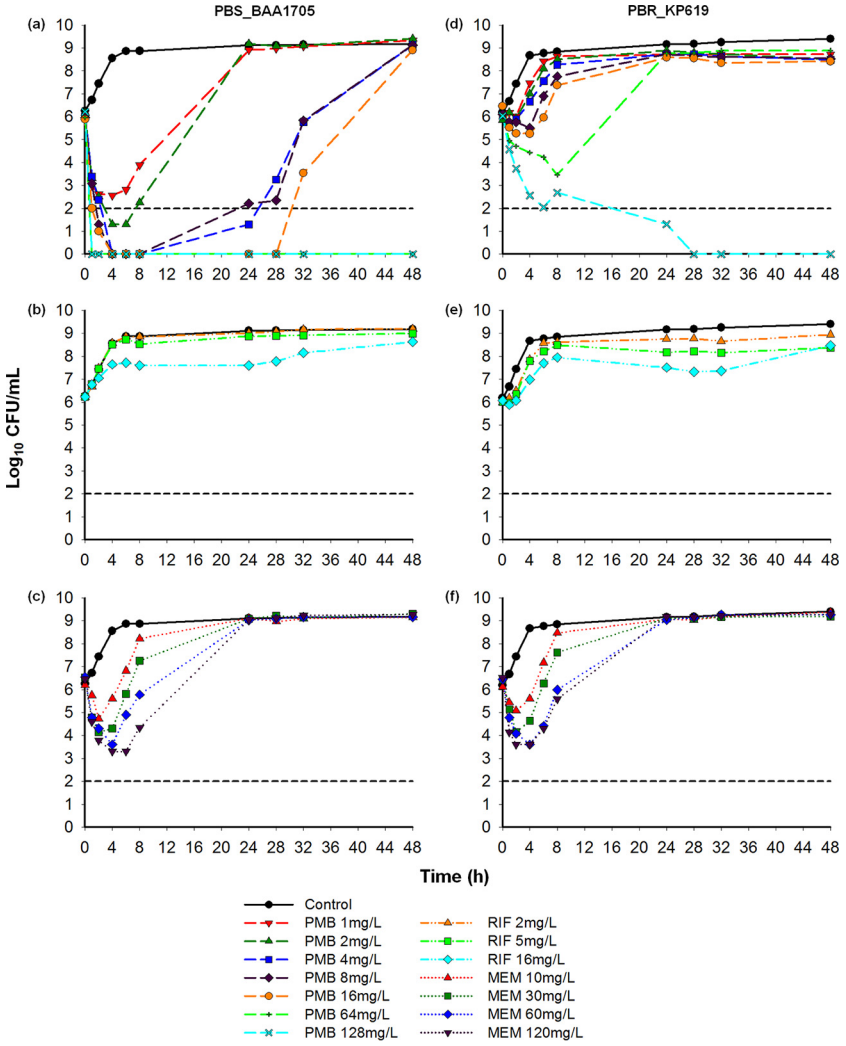

FIG 2 Time-kill curves with various concentrations of polymyxin B (a and d), rifampin (b and e), and meropenem (c and f) alone against an inoculum of ~10^6 CFU/ml of polymyxin-sensitive KPC isolate PBS_BAA1705 (left) and polymyxin-resistant KPC isolate PBR_KP619 (right). PMB, polymyxin B; RIF, rifampin; MEM, meropenem. The limit of quantification is indicated by black dashed horizontal lines.

PBR_KP619 and related pharmacodynamic analyses are shown in Fig. 2 and Table S1 in the supplemental material. Against PBS_BAA1705, all polymyxin B concentrations resulted in early bactericidal activity by 2 h. However, clinically relevant concentrations (unbound C_{ss} values of 1 and 2 mg/liter) resulted in regrowth similar to that of the growth control by 24 h, while 4, 8, and 16 mg/liter polymyxin B resulted in regrowth by 48 h (Fig. 2a). Against PBR_KP619, clinically relevant concentrations of polymyxin B performed similarly to the growth control, and only the highest polymyxin B concentration of 128 mg/liter resulted in bactericidal activity over 48 h (Fig. 2d). Rifampin was ineffective against both isolates and performed similarly to the growth control (Fig. 2b and e). Meropenem monotherapy demonstrated early activity against both isolates followed by regrowth beyond 6 h; the highest concentrations of 60 and 120 mg/liter resulted in a >2-log reduction prior to regrowth (Fig. 2c and f).

(ii) Double-combination therapy. The time-kill curves and pharmacodynamic analyses of polymyxin B-based combinations with rifampin or meropenem are shown in Fig. 3 and Tables 1 and 2. Polymyxin B in combination with rifampin against PBS_BAA1705 resulted in increased killing activity and delayed regrowth compared to monotherapy. Polymyxin B at 1, 2, and 4 mg/liter in combination with all rifampin concentrations

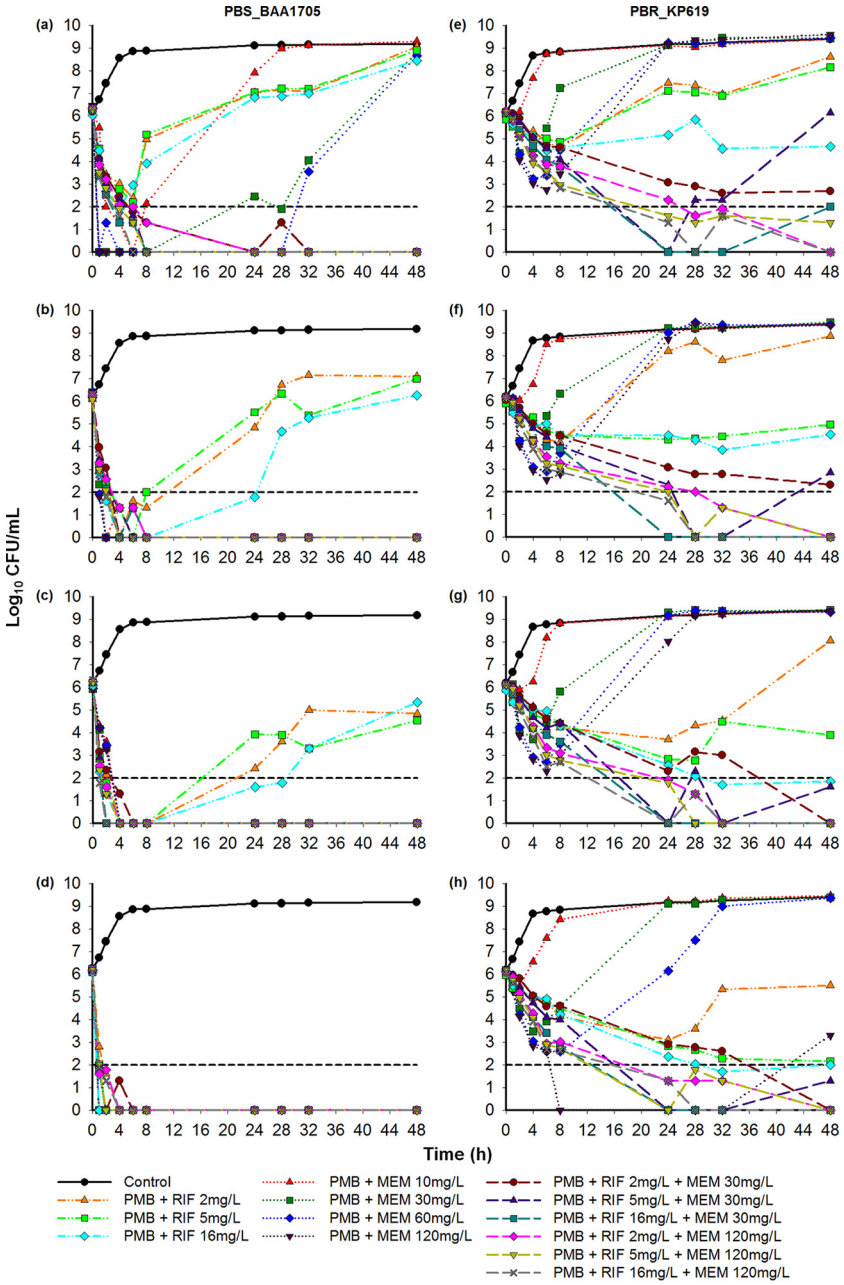

FIG 3 Time-kill curves of polymyxin B-based double combinations with rifampin or meropenem and the triple combination of polymyxin B, rifampin, and meropenem against an inoculum of ~10^6 CFU/ml of polymyxin-sensitive KPC isolate PBS_BAA1705 (left) and polymyxin-resistant KPC isolate PBR_KP619 (right). Polymyxin B-based double and triple combinations are presented according to the following polymyxin B concentrations: polymyxin B at 1 mg/liter (a and e), polymyxin B at 2 mg/liter (b and f), polymyxin B at 4 mg/liter (c and g), and polymyxin B at 8 mg/liter (d and h). PMB, polymyxin B; RIF, rifampin; MEM, meropenem. The limit of quantification is indicated by black dashed horizontal lines.

showed early bactericidal activity followed by regrowth of ≥5 $\log_{10}$ CFU/ml by 48 h (Fig. 3a to c). Polymyxin B at 8 mg/liter in combination with rifampin resulted in sustained killing, with no bacteria being detected beyond 2 h (Fig. 3d). Synergy was observed with polymyxin B at ≥2 mg/liter in combination with rifampin at all concentrations (Table 1). Against PBR_KP619, all polymyxin B and rifampin concentrations in combination were synergistic and resulted in a >1-log reduction by 8 h (Fig. 3e to h and Table 1). Polymyxin B at 4 and 8 mg/liter in combination with rifampin at 5 or 16 mg/liter was bactericidal by 24 h (Fig. 3g and h); however,

TABLE 1 Changes in $\log_{10}$ CFU per milliliter at 4, 8, 24, and 48 h during time-kill experiments with polymyxin B in combination with rifampin against PBS_BAA1705 and PBR_KP619 at an initial inoculum of $\sim 10^6$ CFU/ml[a]

Strain	Time (h)	Change in $\log_{10}$ CFU/ml											
			PMB at 1 mg/liter plus RIF at:			PMB at 2 mg/liter plus RIF at:			PMB at 4 mg/liter plus RIF at:			PMB at 8 mg/liter plus RIF at:		
		Control	2 mg/liter	5 mg/liter	16 mg/liter	2 mg/liter	5 mg/liter	16 mg/liter	2 mg/liter	5 mg/liter	16 mg/liter	2 mg/liter	5 mg/liter	16 mg/liter
PBS_BAA1705	4	2.30	**−3.10**	**−3.38**	**−4.14**	**−6.08**	**−4.88**	**−6.18**	**−6.16**	**−6.08**	**−6.08**	**−6.18**	**−6.11**	**−6.09**
	8	2.61	−1.13	−0.97	−2.14	**−4.78**	**−4.18**	**−6.18**	**−6.16**	**−6.08**	**−6.08**	**−6.18**	**−6.11**	**−6.09**
	24	2.86	0.92	0.89	0.78	−1.25	−0.66	**−4.40**	**−3.75**	−2.16	**−4.48**	**−6.18**	**−6.11**	**−6.09**
	48	2.87	2.95	2.73	2.41	1.00	0.81	0.08	−1.33	−1.55	−0.74	**−6.18**	**−6.11**	**−6.09**
PBR_KP619	4	2.48	−0.79	−0.82	−1.03	−0.94	−0.62	−1.27	−0.75	−1.20	−0.86	−1.16	−0.95	−1.13
	8	2.66	−1.53	−1.01	−1.54	−2.00	−1.41	−1.69	−1.67	−1.58	−1.56	−2.02	−1.46	−1.90
	24	2.97	1.32	1.25	−0.97	2.02	−1.60	−1.69	−2.20	**−3.14**	**−3.28**	**−3.06**	**−3.12**	**−3.77**
	48	3.21	2.47	2.29	−1.48	2.66	−0.94	−1.65	2.16	−2.08	**−4.02**	−0.67	**−3.81**	**−4.13**

[a]Additivity (1- to <2-$\log_{10}$ CFU/ml greater reduction) and synergy (≥2-$\log_{10}$ CFU/ml greater reduction) with the combination compared to the most active single agent in the combination are highlighted in light gray and dark gray, respectively. Boldface type indicates bactericidal activity (≥3-$\log_{10}$ CFU/ml reduction compared to the initial inoculum). PMB, polymyxin B; RIF, rifampin.

TABLE 2 Change in $\log_{10}$ CFU per milliliter at 4, 8, 24, and 48 h during time-kill experiments with polymyxin B in combination with meropenem against PBS_BAA1705 and PBR_KP619 at an initial inoculum of ~10^6 CFU/ml[a]

		Change in $\log_{10}$ CFU/ml															
		PMB at 1 mg/liter plus MEM at:				PMB at 2 mg/liter plus MEM at:				PMB at 4 mg/liter plus MEM at:				PMB at 8 mg/liter plus MEM at:			
Strain	Time (h)	10 mg/liter	30 mg/liter	60 mg/liter	120 mg/liter	10 mg/liter	30 mg/liter	60 mg/liter	120 mg/liter	10 mg/liter	30 mg/liter	60 mg/liter	120 mg/liter	10 mg/liter	30 mg/liter	60 mg/liter	120 mg/liter
PBS_BAA1705	4	**−4.99**	**−6.40**	**−6.35**	**−6.43**	**−4.93**	**−6.16**	**−6.02**	**−6.15**	**−5.91**	**−5.92**	**−5.95**	**−5.92**	**−6.23**	**−6.09**	**−6.16**	**−6.24**
	8	**−4.14**	**−6.40**	**−6.35**	**−6.43**	**−6.24**	**−6.16**	**−6.02**	**−6.15**	**−5.91**	**−5.92**	**−5.95**	**−5.92**	**−6.23**	**−6.09**	**−6.16**	**−6.24**
	24	1.62	**−3.95**	**−6.35**	**−6.43**	**−6.24**	**−6.16**	**−6.02**	**−6.15**	**−5.91**	**−5.92**	**−5.95**	**−5.92**	**−6.23**	**−6.09**	**−6.16**	**−6.24**
	48	3.00	2.38	2.30	**−6.43**	**−6.24**	**−6.16**	**−6.02**	**−6.15**	**−5.91**	**−5.92**	**−5.95**	**−5.92**	**−6.23**	**−6.09**	**−6.16**	**−6.24**
PBR_KP619	4	1.62	−1.61	−2.96	**−3.12**	0.72	−1.90	**−3.12**	**−3.18**	0.12	−2.40	**−3.26**	**−3.35**	0.55	−2.62	**−3.09**	**−3.30**
	8	2.78	1.06	−1.68	−2.68	2.70	0.14	−2.50	**−3.34**	2.69	−0.31	−2.65	**−3.15**	2.40	−1.52	**−3.53**	**−6.12**
	24	3.05	2.96	3.05	3.04	3.07	3.03	2.82	2.61	2.95	3.20	3.00	1.86	3.21	3.01	0.02	**−6.12**
	48	3.33	3.25	3.26	3.47	3.40	3.29	3.15	3.23	3.21	3.29	3.16	3.21	3.45	3.28	3.23	−2.82

[a]Additivity (1- to <2-$\log_{10}$ CFU/ml greater reduction) and synergy (≥2-$\log_{10}$ CFU/ml greater reduction) with the combination compared to the most active single agent in the combination are highlighted in light gray and dark gray, respectively. Boldface type indicates bactericidal activity (≥3-$\log_{10}$ CFU/ml reduction compared to the initial inoculum). PMB, polymyxin B; MEM, meropenem.

sustained bactericidal activity was seen only with polymyxin B at 4 mg/liter combined with rifampin at 16 mg/liter and with polymyxin B at 8 mg/liter with rifampin at 5 or 16 mg/liter (Fig. 3h).

Polymyxin B-based combinations with meropenem against PBS_BAA1705 demonstrated synergy at all concentrations (Table 2). Polymyxin B at 1 mg/liter in combination with meropenem at 10, 30, and 60 mg/liter was bactericidal by 2 h but led to regrowth similar to that of the growth control by 48 h (Fig. 3a). Colonies were undetectable beyond 4 h with polymyxin B at 2, 4, and 8 mg/liter in combination with all concentrations of meropenem (Fig. 3b to d). Polymyxin B in combination with meropenem demonstrated less activity against PBR_KP619 than against PBS_BAA1705 and increased initial killing compared to that of polymyxin in combination with rifampin, but activity was attenuated by 24 h (Fig. 3e to h). Polymyxin B with meropenem at concentrations of $\geq$60 mg/liter resulted in a $>$3-log reduction by 4 h (Fig. 3e to h). Regrowth similar to that of the growth control was seen between 24 and 32 h, whereas polymyxin B at 8 mg/liter combined with meropenem at 120 mg/liter resulted in regrowth of $\sim$3 $\log_{10}$ CFU/ml beyond 24 h (Fig. 3h).

(iii) Triple-combination therapy. Time-kill curves and data from pharmacodynamic analyses of polymyxin B, rifampin, and meropenem concentrations evaluated as a triple combination are shown in Fig. 3 and Tables 3 and 4. All concentrations of the triple combination against PBS_BAA1705 resulted in early bactericidal activity by 4 h and undetectable bacterial counts beyond 8 h (Fig. 3a to d). Synergy was seen only with polymyxin B at 1 mg/liter in combination with meropenem at 30 mg/liter and rifampin at 2, 5, or 16 mg/liter (Table 3). Against PBR_KP619, triple combinations with meropenem at 30 mg/liter resulted in sustained bactericidal activity beyond 24 h, with the exception of polymyxin B at 1 mg/liter in combination with meropenem at 30 mg/liter and rifampin at 5 mg/liter, which led to regrowth close to baseline levels by 48 h (Fig. 3e to h). All triple combinations with meropenem at 120 mg/liter were bactericidal beyond 8 h (Fig. 3e to h). Synergy against PBR_KP619 was observed at all concentrations (Table 4).

(iv) IVDIM. Based on the effectiveness of the triple combination seen in time-kill studies, we further investigated the effect of dynamic clinically relevant concentrations on bacterial killing and the emergence of resistance using an IVDIM. All simulated triple-combination regimens in the IVDIM resulted in a $>$99.9% reduction of the initial inoculum of $\sim$7 $\log_{10}$ CFU/ml for both isolates by 8 h and sustained activity for 48 h (Fig. 4; see also Table S2 in the supplemental material). Against PBS_BAA1705, higher polymyxin B concentrations resulted in increased early activity, shortening the time to sustained bactericidal activity by 2 h for polymyxin B at 1 mg/liter and by 4 h for polymyxin B at 2 mg/liter compared to that for polymyxin B at 0.5 mg/liter (Fig. 4a and b). However, increasing polymyxin B concentrations against PBR_KP619 did not result in a substantial increase in the observed killing activity. A more intensive rifampin regimen with 600 mg every 8 h versus every 12 h provided additional killing activity ($\sim$2-log greater reduction) against PBS_BAA1705 (Fig. 4a and b); however, against PBR_KP619, there was no notable difference (Fig. 4c and d). Regimens simulating an extended infusion (EI) of 2 g meropenem every 8 h did not reveal a consistent trend of increased activity compared to the activity with 1 g every 8 h.

Population analysis profiles (PAPs) of PBS_BAA1705 (MIC, 0.5 mg/liter) did not contain resistant subpopulations in the presence of polymyxin B concentrations of $>$1 mg/liter at baseline or 24 or 48 h after exposure to triple-combination regimens in the IVDIM. In the case of PBR_KP619 (MIC, 64 mg/liter), resistant subpopulations were not observed in the presence of polymyxin B at concentrations of $>$64 mg/liter at baseline or at 24 or 48 h. After exposure to clinically relevant polymyxin B concentrations of 0.5, 1, and 2 mg/liter as a part of the triple-combination regimens evaluated over 48 h, emergence of polymyxin B resistance was not seen for either isolate (see Fig. S1 and S2 in the supplemental material).

TABLE 3 Change in $\log_{10}$ CFU per milliliter at 4, 8, 24, and 48 h during time-kill experiments with polymyxin B in triple combination with rifampin and meropenem at 30 mg/liter against PBS_BAA1705 and PBR_KP619 at an initial inoculum of ~10^6 CFU/ml[a]

		Change in $\log_{10}$ CFU/ml											
			PMB at 1 mg/liter, MEM at 30 mg/liter, and RIF at:			PMB at 2 mg/liter, MEM at 30 mg/liter, and RIF at:			PMB at 4 mg/liter, MEM at 30 mg/liter, and RIF at:			PMB at 8 mg/liter, MEM at 30 mg/liter, and RIF at:		
Strain	Time (h)	Control	2 mg/liter	5 mg/liter	16 mg/liter	2 mg/liter	5 mg/liter	16 mg/liter	2 mg/liter	5 mg/liter	16 mg/liter	2 mg/liter	5 mg/liter	16 mg/liter
PBS_BAA1705	4	2.30	**−3.74**	**−4.02**	**−4.96**	**−6.23**	**−4.96**	**−6.31**	**−4.97**	**−6.23**	**−6.21**	**−4.92**	**−6.14**	**−6.24**
	8	2.61	**−4.89**	**−6.37**	**−6.26**	**−6.23**	**−6.26**	**−6.31**	**−6.27**	**−6.23**	**−6.21**	**−6.23**	**−6.14**	**−6.24**
	24	2.86	**−6.19**	**−6.37**	**−6.26**	**−6.23**	**−6.26**	**−6.31**	**−6.27**	**−6.23**	**−6.21**	**−6.23**	**−6.14**	**−6.24**
	48	2.87	**−6.19**	**−6.37**	**−6.26**	**−6.23**	**−6.26**	**−6.31**	**−6.27**	**−6.23**	**−6.21**	**−6.23**	**−6.14**	**−6.24**
PBR_KP619	4	2.48	−1.09	−1.07	−1.49	−1.03	−1.34	−1.92	−1.07	−1.55	−1.94	−1.09	−1.37	−2.06
	8	2.66	−1.50	−2.05	−2.39	−1.56	−2.15	−2.26	−1.76	−1.81	−2.54	−1.53	−2.11	**−3.35**
	24	2.97	**−3.05**	**−6.13**	**−6.17**	−2.96	**−3.85**	**−6.16**	**−3.88**	**−6.22**	**−6.14**	**−3.23**	**−6.11**	**−6.17**
	48	3.21	**−3.45**	0.02	**−4.17**	**−3.74**	**−3.30**	**−6.16**	**−6.18**	**−4.62**	**−6.14**	**−6.13**	**−4.81**	**−6.17**

[a]Additivity (1- to <2-$\log_{10}$ CFU/ml greater reduction) and synergy (≥2-$\log_{10}$ CFU/ml greater reduction) with the triple combination compared to the most active duo in the combination are highlighted in light gray and dark gray, respectively. Boldface type indicates bactericidal activity (≥3-$\log_{10}$ CFU/ml reduction compared to the initial inoculum). PMB, polymyxin B; RIF, rifampin; MEM, meropenem.

TABLE 4 Change in $\log_{10}$ CFU per milliliter at 4, 8, 24, and 48 h during time-kill experiments with polymyxin B in triple combination with rifampin and meropenem at 120 mg/liter against PBS_BAA1705 and PBR_KP619 at an initial inoculum of $\sim 10^6$ CFU/ml[a]

		Change in $\log_{10}$ CFU/ml											
			PMB at 1 mg/liter, MEM at 120 mg/liter, and RIF at:			PMB at 2 mg/liter, MEM at 120 mg/liter, and RIF at:			PMB at 4 mg/liter, MEM at 120 mg/liter, and RIF at:			PMB at 8 mg/liter, MEM at 120 mg/liter, and RIF at:		
Strain	Time (h)	Control	2 mg/liter	5 mg/liter	16 mg/liter	2 mg/liter	5 mg/liter	16 mg/liter	2 mg/liter	5 mg/liter	16 mg/liter	2 mg/liter	5 mg/liter	16 mg/liter
PBS_BAA1705	4	2.30	**−4.24**	**−4.39**	**−4.72**	**−4.96**	**−6.08**	**−6.30**	**−6.25**	**−6.23**	**−6.32**	**−6.18**	**−6.19**	**−6.09**
	8	2.61	**−5.02**	**−6.30**	**−6.33**	**−6.26**	**−6.08**	**−6.30**	**−6.25**	**−6.23**	**−6.32**	**−6.18**	**−6.19**	**−6.09**
	24	2.86	**−6.32**	**−6.30**	**−6.33**	**−6.26**	**−6.08**	**−6.30**	**−6.25**	**−6.23**	**−6.32**	**−6.18**	**−6.19**	**−6.09**
	48	2.87	**−6.32**	**−6.30**	**−6.33**	**−6.26**	**−6.08**	**−6.30**	**−6.25**	**−6.23**	**−6.32**	**−6.18**	**−6.19**	**−6.09**
PBR_KP619	4	2.48	−1.94	−2.29	−1.97	−1.94	−1.93	−2.25	−1.83	−1.94	−2.31	−1.82	−2.09	−2.20
	8	2.66	−2.42	**−3.23**	**−3.30**	−2.90	**−3.05**	**−3.26**	**−3.01**	**−3.35**	**−3.42**	**−3.06**	**−3.44**	**−3.46**
	24	2.97	**−3.90**	**−4.59**	**−4.82**	**−3.95**	**−4.17**	**−4.54**	**−4.20**	−4.35	**−6.12**	**−4.78**	**−6.16**	**−4.81**
	48	3.21	**−6.20**	**−4.89**	**−6.12**	**−6.16**	**−6.17**	**−6.14**	**−6.10**	**−6.13**	**−6.12**	**−6.08**	**−6.16**	**−6.11**

[a]Additivity (1- to <2-$\log_{10}$ CFU/ml greater reduction) and synergy (≥2-$\log_{10}$ CFU/ml greater reduction) with the triple combination compared to the most active duo in the combination are highlighted in light gray and dark gray, respectively. Boldface type indicates bactericidal activity (≥3-$\log_{10}$ CFU/ml reduction compared to the initial inoculum). PMB, polymyxin B; RIF, rifampin; MEM, meropenem.

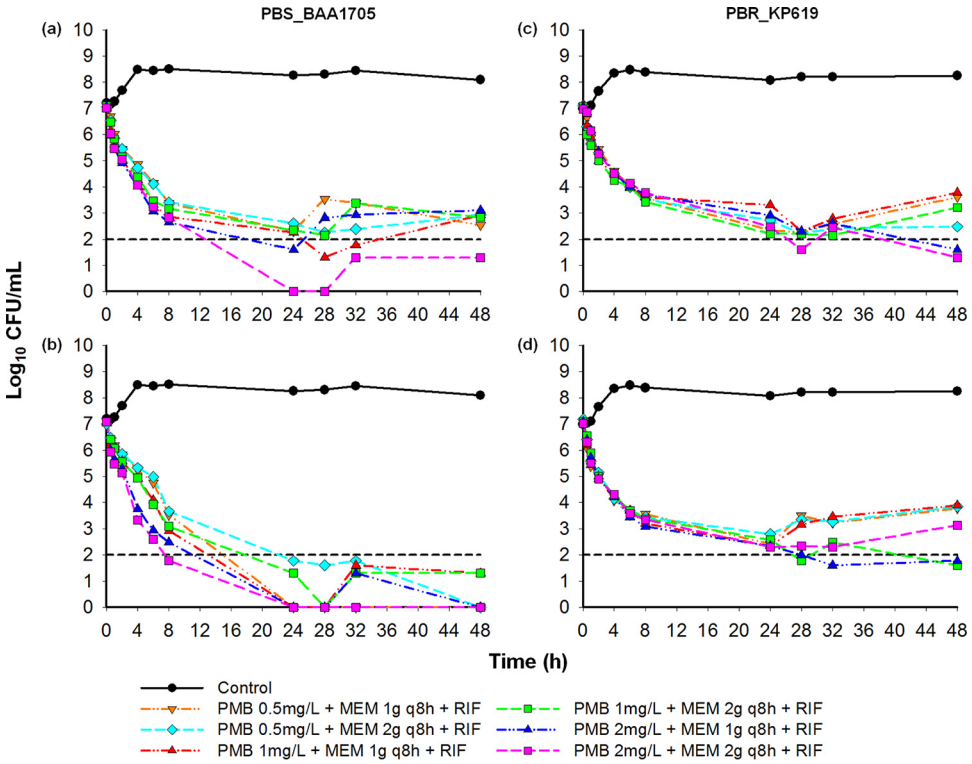

FIG 4 Time course of the change in the bacterial density in response to triple-antibiotic regimens with polymyxin B, rifampin, and meropenem against an inoculum of ~10^7 CFU/ml of polymyxin-sensitive KPC isolate PBS_BAA1705 (left) and polymyxin-resistant KPC isolate PBR_KP619 (right) in a dynamic one-compartment infection model. Shown are data for rifampin at 600 mg dosed every 12 h (a and c) and rifampin at 600 mg dosed every 8 h (b and d). PMB, polymyxin B; RIF, rifampin; MEM, meropenem; q8h, every 8 h. The limit of quantification is indicated by the black dashed horizontal line.

DISCUSSION

The escalating prevalence of infections caused by multidrug-resistant (MDR) Gram-negative bacteria with limited therapeutic options has led to the revival of polymyxins. KPC enzyme expression, a common mechanism of resistance among carbapenem-resistant *Enterobacteriaceae*, has further compounded the antibiotic resistance crisis, resulting in the increased use of polymyxins (22, 23). Recently, increasing numbers of reports of polymyxin resistance in strains that were previously susceptible have clinicians worried, as they could potentially be faced with the challenge of having virtually no therapeutic alternatives (25).

In the present study, we systematically evaluated the *in vitro* activities of polymyxin B, rifampin, and meropenem alone and in combination against PB^s and PB^r KPC-producing *K. pneumoniae* isolates. Monotherapy with these antibiotics was ineffective. Although polymyxin B showed early bactericidal activity against the PB^s strain, the marked regrowth seen by 24 h is suggestive of selection for resistance and the potential for clinical failure. Several studies have evaluated treatment outcomes against infections caused by KPC-producing *K. pneumoniae* isolates, and despite the susceptibility profiles of these isolates, monotherapy with an active antibiotic is associated with greater mortality than combination therapy (8, 26, 27).

The pharmacodynamic activity of polymyxin B with rifampin was increased compared to that with monotherapy with either agent against both isolates. This is consistent with previous *in vitro* studies that indicated synergy between polymyxins and rifampin against KPC-producing *K. pneumoniae* (21–24). However, beyond 24 h, our analyses revealed that clinically achievable free-drug concentrations were unable to provide a sustained reduction in the bacterial burden. Polymyxin B with meropenem against the PB^s isolate resulted in more extensive and sustained killing than did

polymyxin B with rifampin. This is in agreement with data from other *in vitro* studies that evaluated PB^s KPC-producing *K. pneumoniae* isolates and detected synergy between polymyxin B and meropenem (21, 28). The combination of polymyxin B and meropenem has also been shown to significantly reduce mortality in infected rats compared to either agent as monotherapy (29).

Several retrospective observational studies evaluating clinical outcomes of patients with infections caused by KPC-producing *K. pneumoniae* have shown that carbapenem-containing combinations are associated with lower mortality rates (18, 30). While the combination of polymyxin and meropenem appears to be effective, there is a paucity of data regarding the efficacy of this combination in the presence of polymyxin resistance. Our data suggest that these two-drug combinations lack sustained activity against highly PB^r strains.

Tangden et al. evaluated colistin, rifampin, and meropenem against PB^s (colistin MIC, 0.125 mg/liter) metallo-beta-lactamase-producing *K. pneumoniae* in static time-kill studies and found that this triple combination was synergistic and bactericidal over 24 h (31). Furthermore, the combination of polymyxin B, rifampin, and doripenem was bactericidal over 24 h against PB^s (polymyxin B MIC, 0.75 to 1 mg/liter) KPC-producing *K. pneumoniae* with concentrations at one-quarter the MICs (32). The time-kill results for the combination of polymyxin B, rifampin, and meropenem presented here are consistent with those findings and further demonstrate the effectiveness of this triple combination over 48 h against PB^r (polymyxin B MIC, 64 mg/liter) KPC-producing *K. pneumoniae*. Synergy was found at all concentrations of polymyxin B, rifampin, and meropenem in combination against the PB^r isolate. Clinical data regarding the use of this triple combination are limited. Biancofiore et al. reported the successful treatment of a case of multifocal infection due to an MDR *Acinetobacter baumannii* strain susceptible only to colistin (MIC, 1 mg/liter) by using colistin (2 million units twice daily), rifampin (600 mg daily), and meropenem (1 g three times daily) in combination over 24 days (33).

To the best of our knowledge, this is the first study to use an IVDIM to evaluate clinically relevant triple-combination regimens of polymyxin B, rifampin, and meropenem against *K. pneumoniae* over 48 h and to assess their effects on the emergence of resistance. All simulated regimens comprising polymyxin B (C_{ss}, 0.5, 1, and 2 mg/liter), rifampin at 600 mg dosed every 8 or 12 h, and meropenem at 1 or 2 g as an extended 3-h infusion every 8 h were effective against the resistant isolate. Interestingly, the triple combinations in the present study were bactericidal despite the MIC of each constituent antibiotic being well above the susceptibility breakpoint. The synergy seen here may be due to the antibiotics having different molecular targets. Binding of polymyxin B to lipopolysaccharide on the bacterial outer membrane results in rapid permeabilization and enhanced penetration by polymyxin B, meropenem, and rifampin, allowing for increased binding to the inner membrane, penicillin-binding protein, and RNA polymerase, respectively (34). Additionally, given the loss of cell wall integrity caused by meropenem binding, meropenem can further enhance the access of polymyxin B and rifampin to their target sites and increase pharmacodynamic activity.

It is important to acknowledge the potential limitations of our study. First, the *in vitro* experiment duration of 48 h may not be long enough to observe a change in resistance profiles or discern differences between regimens with respect to the emergence of resistance. Second, two strains were evaluated, and the PB^s and PB^r clinical isolates may not be representative of all KPC-producing *K. pneumoniae* isolates. Finally, the *in vitro* system is a simplification of the *in vivo* scenario, and despite its advantages, the killing activity reported here is in the absence of a host immune system.

The use of combination therapy with more than two agents is a widely accepted practice for the treatment of infectious diseases such as tuberculosis or human immunodeficiency virus infections, but this approach is not commonplace for the treatment of infections by MDR Gram-negative bacteria. Safety is of particular concern with regimens containing multiple antibiotics and intensive dosing. Clinical trials to evaluate dosing implications of these triple combinations against resistant pathogens would be

beneficial to assess effective treatment options and enable dose optimization in terms of safety and efficacy. In addition, studies to evaluate innovative polymyxin dosing strategies (such as with front loading [35]) will enable the design of novel regimens with effective and sustained pharmacodynamic activity by managing polymyxin exposure and minimizing the risk of dose-dependent nephrotoxicity, a concern with polymyxin use (36, 37).

In conclusion, we demonstrate for the first time that antibiotic regimens consisting of polymyxin B, rifampin, and meropenem may be viable treatment options against PB^r KPC-producing *K. pneumoniae* isolates. Further evaluation of these regimens against an array of isolates expressing resistance-promoting genes and over an extended period of time is warranted to optimize triple-combination therapy, especially in an age of emergent polymyxin resistance.

MATERIALS AND METHODS

Bacterial strains. Two strains of *K. pneumoniae* were utilized in this study: KP619, a clinical blood isolate obtained from a patient at the Kingman Regional Medical Center, Kingman, AZ, and BAA1705, a clinical strain obtained from the ATCC. Both isolates are KPC-2 producers and resistant to carbapenems and rifampin (meropenem MIC of 64 mg/liter and rifampin MIC of 64 mg/liter for both). KP619 has a nonsense mutation in *mgrB* and is PB^r (PBR_KP619) (polymyxin B MIC, 64 mg/liter). BAA1705 has a wild-type *mgrB* sequence and is PB^s (PBS_BAA1705) (polymyxin B MIC, 0.5 mg/liter). MICs were determined in triplicate by broth microdilution according to Clinical and Laboratory Standards Institute guidelines (54). PCR was performed by using previously described primer sets for β-lactamase Ambler classes A (GES and KPC), B (NDM, VIM, and IMP), and D (OXA48 and -40) (38) and for *mgrB* (39). Genomic DNA was extracted from bacterial isolates by using the EZNA bacterial DNA kit (Omega Biotek, Norcross, GA). PCR products were analyzed by gel electrophoresis and sequenced (Roswell Park Cancer Institute, Buffalo, NY). Nucleotide and deduced protein sequences were analyzed by using the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/).

Antimicrobials and media. Mueller-Hinton broth (Becton, Dickinson and Company, Sparks, MD) supplemented with calcium and magnesium (25.0 mg/liter Ca^{2+} and 12.5 mg/liter Mg^{2+}) (CAMHB) was used for susceptibility testing and all *in vitro* models. Stock solutions of polymyxin B (lot number WXBB4470V; Sigma-Aldrich, St. Louis, MO) and meropenem (lot number LC24337; AK Scientific, Union City, CA) were freshly prepared in sterile water and saline prior to each experiment. Rifampin (lot number 141157; Fisher Scientific, Fair Lawn, NJ) was dissolved in a minimal amount of methanol before dilution with sterile saline. All drug solutions were filter sterilized by using a 0.22-μm filter (Fisher Scientific, Pittsburgh, PA).

Static time-kill kinetics. Static time-kill experiments were performed over 48 h to evaluate the rate and extent of killing by polymyxin B (1, 2, 4, 8, 16, 64, and 128 mg/liter), rifampin (2, 5, and 16 mg/liter), and meropenem (10, 30, 60, and 120 mg/liter) alone and by polymyxin B-based combinations with rifampin or meropenem. Additionally, a 4-by-3-by-2 concentration array of the triple combination of polymyxin B (1, 2, 4, and 8 mg/liter), rifampin (2, 5, and 16 mg/liter), and meropenem (30 and 120 mg/liter) was evaluated against both isolates. Concentrations were selected to assess a broad range of free-drug concentrations, including clinically achievable and higher concentrations, for dose optimization using the one-compartment model (40–46). Antibiotics were added to a logarithmic-phase broth culture prepared prior to each experiment by adding fresh bacterial colonies grown overnight to prewarmed CAMHB (37°C) to achieve the desired initial inoculum of $\sim 10^6$ CFU/ml. Serial samples were obtained at 0, 1, 2, 4, 6, 8, 24, 28, 32, and 48 h for quantification of bacteria.

***In vitro* dynamic infection model.** A one-compartment PK/PD IVDIM described previously (35) was used to simulate different triple-combination regimens against an initial inoculum of $\sim 10^7$ CFU/ml over 48 h. A fresh bacterial stock was injected into the central compartment to achieve the desired inoculum. The temperature of the central compartment was maintained at 37°C with constant stirring to ensure homogeneous mixing and instantaneous distribution. A peristaltic pump (Masterflex L/S; Cole-Parmer, Vernon Hills, IL), with a flow rate of 1.56 ml/min to simulate a half-life ($t_{1/2}$) of 2 h for rifampin and meropenem, was used to deliver CAMHB into the central compartment with displacement of an equal volume (43, 47). Polymyxin B was administered as a constant infusion of 0.5, 1, or 2 mg/liter into the central compartment throughout the experiment (area under the concentration-time curve to 24 h [AUC_{24}] of 12, 24, or 48 mg · h/liter), thereby simulating the unbound average steady-state concentrations (C_{ss}) and flat concentration-time profiles seen in critically ill patients (40, 41, 48). Rifampin regimens of 600 mg dosed intravenously every 8 h and every 12 h were simulated by using an automated syringe pump to inject the drug into the central compartment to achieve a free peak concentration (C_{max}) of 5 mg/liter (42, 43, 48). We simulated the current clinical dosing strategy of an initial meropenem bolus followed by an EI of meropenem (44–46). Meropenem was injected into the central compartment following bacterial inoculation to attain a C_{max} of 120 mg/liter to simulate a meropenem bolus of 1 g over 10 min or of 2 g over 30 min. An EI of meropenem over 3 h dosed every 8 h was achieved by using a multichannel syringe pump (New Era Pump Systems, Farmingdale, NY) to attain an unbound steady-state C_{max} of 40 or 80 mg/liter, simulating a 3-h EI of 1 or 2 g dosed every 8 h, respectively (44). Simulated doses were selected to assess a range of clinically tolerable regimens based on data from previously reported clinical studies (40–46) and to evaluate potential benefits of intensive dosing (polymyxin B

AUC_{24} of 48 mg · h/liter, rifampin at 600 mg with a dosing interval of 8 h, and meropenem at 2 g with a dosing interval of 8 h) for bacterial killing and suppression of resistance. Serial samples were obtained at 0, 0.5, 1, 2, 4, 6, 8, 24, 28, 32, and 48 h for quantification of bacteria. Additionally, three sets of samples (500 μl) were stored at −80°C until pharmacokinetic validation.

Quantification of bacteria and population analysis profiles. All bacterial samples were serially diluted with sterile saline and plated (50 μl) onto Mueller-Hinton II agar (MHA; Becton, Dickinson and Company, Sparks, MD) plates by using Whitley Automated Spiral Plater II (Don Whitley Scientific, West Yorkshire, UK). Colony counts ($\log_{10}$ CFU per milliliter) were quantified by using the ProtoCOL HR automated bacterial colony counter (Synbiosis, Frederick, MD) after 24 h of incubation at 37°C; the limit of quantification was 2 $\log_{10}$ CFU/ml. To assess for the emergence of polymyxin B resistance, PAPs were determined by plating samples collected at 0 h (baseline), 24 h, and 48 h onto polymyxin B-containing MHA (1, 2, 4, 8, 16, 64, 128, and 256 mg/liter) for all regimens evaluated by using the IVDIM.

Pharmacokinetic validation. All samples were analyzed within 4 weeks of their collection. Polymyxin B1 and B2 concentrations were quantified by using a validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay (49). Analysis of independently prepared quality control samples indicated good reproducibility (coefficients of variation of ≤7.89%) and accuracy (measured concentrations that were ≤10.5% from target concentrations). The limit of quantification was 0.025 mg/liter. Rifampin and meropenem concentrations were quantified by high-performance liquid chromatography (HPLC) methods as previously described and modified for CAMHB (50, 51). The assays were linear ($r^2 >$ 0.999) over concentrations from 1 to 15 mg/liter and from 5 to 150 mg/liter, respectively.

Pharmacodynamic analysis. The pharmacodynamic effect was quantified as the change in $\log_{10}$ CFU per milliliter at time t (CFU_t) (4, 8, 24, and 48 h) compared to the baseline value (0 h) (CFU_0) as follows: log change = $\log_{10}(CFU_t) - \log_{10}(CFU_0)$. Bactericidal activity was defined as a ≥3-$\log_{10}$ CFU/ml reduction compared to the initial inoculum. Additivity and synergy were defined as 1- to <2-$\log_{10}$ CFU/ml and ≥2-$\log_{10}$ CFU/ml greater reductions by the double (or triple) combination than that with the most active single (or dual) agent in the combination, respectively.

SUPPLEMENTAL MATERIAL

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TEXT S1, PDF file, 0.5 MB.

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