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PHARMACOLOGY

Polymyxin Triple Combinations against Polymyxin-Resistant, Multidrug-Resistant, KPC-Producing *Klebsiella pneumoniae*

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ABSTRACT Resistance to polymyxin antibiotics is increasing. Without new antibiotic classes, combination therapy is often required. We systematically investigated bacterial killing with polymyxin-based combinations against multidrug-resistant (including polymyxin-resistant), carbapenemase-producing *Klebsiella pneumoniae*. Monotherapies and double- and triple-combination therapies were compared to identify the most efficacious treatment using static time-kill studies (24 h, six isolates), an *in vitro* pharmacokinetic/pharmacodynamic model (IVM; 48 h, two isolates), and the mouse thigh infection model (24 h, six isolates). In static time-kill studies, all monotherapies (polymyxin B, rifampin, amikacin, meropenem, or minocycline) were ineffective. Initial bacterial killing was enhanced with various polymyxin B-containing double combinations; however, substantial regrowth occurred in most cases by 24 h. Most polymyxin B-containing triple combinations provided greater and more sustained killing than double combinations. Standard dosage regimens of polymyxin B (2.5 mg/kg of body weight/day), rifampin (600 mg every 12 h), and amikacin (7.5 mg/kg every 12 h) were simulated in the IVM. Against isolate ATH 16, no viable bacteria were detected across 5 to 25 h with triple therapy, with regrowth to ~2-$\log_{10}$ CFU/ml occurring at 48 h. Against isolate BD 32, rapid initial killing of ~3.5-$\log_{10}$ CFU/ml at 5 h was followed by a slow decline to ~2-$\log_{10}$ CFU/ml at 48 h. In infected mice, polymyxin B monotherapy (60 mg/kg/day) generally was ineffective. With triple therapy (polymyxin B at 60 mg/kg/day, rifampin at 120 mg/kg/day, and amikacin at 300 mg/kg/day), at 24 h there was an ~1.7-$\log_{10}$ CFU/thigh reduction compared to the starting inoculum for all six isolates. Our results demonstrate that the polymyxin B-rifampin-amikacin combination significantly enhanced *in vitro* and *in vivo* bacterial killing, providing important information for the optimization of polymyxin-based combinations in patients.

KEYWORDS *Klebsiella pneumoniae*, polymyxin resistance, combination, amikacin, rifampin

Klebsiella pneumoniae is a major Gram-negative opportunistic pathogen responsible for nosocomial respiratory tract, bloodstream, and urinary tract infections (1–4). Infections caused by multidrug-resistant (MDR) *K. pneumoniae* have been increasingly reported over the past few years (3, 5, 6), with a mortality rate of up to 40% in patients infected with carbapenemase-producing (KPC) *K. pneumoniae* (7–11). The World Health Organization (WHO) has designated carbapenem-resistant *K. pneumoniae* one of three critical priority pathogens on the list for the research and development of new

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TABLE 1 MICs of KPC-producing *K. pneumoniae* isolates used in this study

Isolate[a]	MIC (μg/ml)				
	PMB[b]	RIF[d]	AMI[b]	MIN[c]	MER[b]
ATH 8	64	16	16	4	4
ATH 16	32	16	16	4	64
ATH 18	64	16	16	4	128
ATH 24	32	16	16	4	64
BD 32	32	128	128	8	64
BD 46	128	32	16	4	64

[a]All isolates were multidrug resistant, defined as nonsusceptible to at least one agent in three or more antimicrobial categories (67). BD 32 additionally was pandrug resistant, defined as nonsusceptible to all agents in all antimicrobial classes (66).
[b]EUCAST breakpoints (S, susceptible; R, resistant) were S $\leq$ 8 μg/ml and R $>$ 8 μg/ml for amikacin and S $\leq$ 2 μg/ml and R $>$ 8 μg/ml for meropenem; the EUCAST breakpoints for colistin of S $\leq$ 2 and R $>$ 2 were applied to polymyxin B (69, 70).
[c]CLSI breakpoints (S, susceptible; R, resistant) were S $\leq$ 4 μg/ml and R $\geq$ 16 μg/ml for minocycline (68).
[d]Rifampin as monotherapy is normally inactive against the *Enterobacteriaceae*, and breakpoints have not been established.

antibiotics (12). Given the critical shortage of effective treatment options for use against infections caused by MDR *K. pneumoniae*, including KPC-producing *K. pneumoniae* strains (13, 14), the old polymyxins (i.e., polymyxin B [PMB] and colistin) are increasingly used in the clinic. Polymyxins are naturally occurring cyclic lipopeptides (15, 16) whose use declined in the 1980s due to reports of nephro- and neurotoxicity (17–19). However, they have attracted significant interest over the last decade, given their activity against many MDR Gram-negative organisms (20, 21). Worryingly, polymyxin-resistant *K. pneumoniae* isolates have been reported both *in vitro* and *in vivo* (21–23). Although a number of *in vitro* studies have demonstrated that polymyxin-based double combinations can exhibit rapid initial killing against polymyxin-susceptible and -resistant MDR *K. pneumoniae* isolates, bacterial regrowth commonly occurs soon after (24, 25). Thus, there is an urgent need to develop novel, rational combinations for the treatment of polymyxin-resistant, MDR *K. pneumoniae* isolates. This *in vitro* and *in vivo* study aimed to evaluate bacterial killing and resistance suppression of polymyxin-based double and triple combinations against polymyxin-resistant, MDR *K. pneumoniae* clinical isolates and to identify the most active triple combination *in vitro* and *in vivo*.

(This study was presented at ASM Microbe, 7 to 11 June 2018.)

RESULTS

MICs and static-concentration time-kill experiments. MICs to each antibiotic are shown in Table 1. All six *K. pneumoniae* isolates were susceptible or intermediately resistant to minocycline (MIN) and resistant to all remaining antibiotics tested, except ATH 8, which was not resistant to meropenem (MER). The results of the static-concentration time-kill experiments are shown in Table 2 (log changes in viable cell counts) and Fig. S1 to S3 in the supplemental material (time-kill curves). All antibiotic monotherapies were ineffective against all isolates across 24 h (maximum killing of 0.7 $\log_{10}$ CFU/ml, with subsequent regrowth; Table 2 and Fig. S1). Polymyxin B with either minocycline or rifampin (RIF) were additive or synergistic against most isolates across 24 h (except polymyxin B plus minocycline against BD 32), with substantially enhanced initial killing (at 1 h) of up to ~4 $\log_{10}$ CFU/ml compared to monotherapy (Table 2 and Fig. S2). Polymyxin B plus either amikacin (AMI) or meropenem showed additivity or synergy against fewer isolates and generally not at 24 h. Other double combinations against all isolates, with the exception of BD 46 at 1 and 4 h, typically were ineffective. Despite the enhanced killing, regrowth was observed with all isolates treated with double antibiotic combinations such that by 24 h, even synergistic combinations had exceeded, or were close to, the initial inoculum.

With the exception of BD 32, where only the combinations of polymyxin B with rifampin plus either amikacin or meropenem enhanced bacterial killing, the polymyxin B-based triple combinations were highly synergistic (up to ~5 $\log_{10}$ CFU/ml greater

TABLE 2 Log changes in viable cell counts at 1, 4, and 24 h with clinically relevant concentrations of PMB, RIF, AMI, MIN, and MER against six MDR *K. pneumoniae* isolates using static time-kill experiments[a]

Treatment	Log change [log10(CFU*t*) - log10(CFU$_0$)]																	
	ATH 8			ATH 16			ATH 18			ATH 24			BD 32			BD 46		
	1h	4h	24h	1h	4h	24h	1h	4h	24h	1h	4h	24h	1h	4h	24h	1h	4h	24h
Control	1.1	1.9	1.6	0.9	1.7	2.5	1.1	1.9	2.2	0.8	1.9	2.4	0.0	1.3	2.2	0.1	0.7	2.0
PMB	0.8	1.4	1.8	0.7	1.9	1.8	0.1	2.0	2.3	0.0	1.6	1.8	0.1	1.1	2.1	0.4	1.0	1.9
RIF	0.9	0.8	1.6	-0.2	-0.1	2.2	-0.5	1.8	1.5	1.2	2.2	3.0	-0.2	1.0	2.1	0.4	0.7	1.8
AMI	-0.2	1.3	1.5	0.6	1.8	1.2	-0.2	0.7	1.7	-0.4	1.1	2.0	-0.2	1.0	2.1	-0.4	-0.7	1.7
MIN	-0.3	-0.6	1.7	-0.3	-0.3	1.6	-0.6	-0.7	1.7	-0.3	-0.5	1.9	-0.1	0.8	1.8	-0.6	-0.6	1.9
MER	0.0	-0.3	1.9	-0.2	0.0	0.1	0.7	0.9	1.8	-0.5	1.4	2.2	-0.9	-0.5	1.8	-0.7	-0.7	1.9
PMB+AMI	**-1.2**	**-1.1**	1.5	**-1.8**	**-1.7**	2.1	-1.0	**-1.2**	2.1	-1.0	**-1.2**	2.0	-0.2	1.2	2.0	**-2.3**	-1.6	**0.4**
PMB+MIN	**-1.6**	**-1.9**	**-0.6**	**-3.2**	**-3.5**	**0.6**	**-2.8**	**-2.3**	**-1.0**	**-2.8**	**-2.6**	**-0.5**	-0.4	**-0.8**	1.9	**-3.1**	**-4.7**	**-0.2**
PMB+RIF	**-3.5**	**-3.7**	**-1.0**	**-1.8**	**-4.0**	**0.6**	**-3.8**	**-3.1**	**0.1**	**-3.9**	**-1.2**	**0.8**	**-1.3**	**-2.2**	**-0.3**	**-3.0**	-1.6	1.0
PMB+MER	**-3.1**	**-2.5**	1.7	0.6	1.9	1.9	0.1	1.9	2.5	**-4.2**	1.6	2.0	-0.5	1.0	2.0	**-4.6**	**-2.7**	1.9
RIF+AMI	-0.4	0.0	2.1	**-2.3**	1.1	1.0	0.7	1.3	1.9	0.3	1.6	2.0	-0.7	0.8	2.2	-1.3	-0.9	1.7
RIF+MIN	-0.4	-0.2	1.8	**-1.3**	**-1.8**	1.4	-0.5	-0.3	2.0	-0.5	-0.6	2.3	-0.3	1.0	2.1	-0.3	**-1.6**	1.7
RIF+MER	-0.4	-0.1	1.8	-0.1	-0.6	1.3	0.3	**-0.1**	1.2	0.4	0.7	1.6	-0.5	-1.1	2.0	**-3.6**	**-3.7**	1.8
MIN+MER	-0.6	-0.5	**0.5**	**-3.5**	-1.2	1.5	**-2.2**	0.1	1.7	**-2.1**	-0.6	2.3	-0.9	-0.7	1.7	**-3.8**	**-4.3**	**0.4**
AMI+MER	-0.6	-0.8	1.5	-0.8	-0.9	1.2	-0.5	**-0.4**	1.5	0.6	0.7	1.3	-0.8	-1.0	1.6	**-2.6**	**-3.8**	1.7
PMB+RIF+AMI	**-3.3**	**-2.8**	**-1.4**	**-5.6**	**-5.6**	**-1.6**	**-4.9**	**-2.7**	**-1.6**	**-5.3**	**-3.5**	**-1.8**	-1.1	**-3.0**	**-4.6**	**-2.3**	**-2.9**	**-2.6**
PMB+AMI+MER	**-1.9**	**-4.3**	**-1.6**	**-2.0**	**-2.6**	**-0.9**	-1.1	**-2.3**	1.5	-0.8	**-2.7**	1.6	-0.9	1.0	1.9	**-5.9**	**-3.7**	1.9
PMB+RIF+MIN	**-4.5**	**-3.7**	**-1.6**	**-5.6**	**-5.6**	**-1.1**	**-4.6**	**-4.1**	1.2	**-4.6**	**-3.1**	**-1.1**	-1.7	**-1.6**	1.4	**-2.7**	**-2.0**	**-1.6**
PMB+RIF+MER	**-2.8**	**-4.3**	**-1.6**	**-3.3**	**-3.5**	**-1.6**	**-4.3**	**-2.6**	0.7	**-3.5**	**-2.4**	**0.7**	-1.3	**-1.9**	**-2.6**	**-3.6**	**-4.6**	**-1.1**
PMB+MIN+MER	**-3.9**	**-4.6**	**-1.9**	**-2.7**	**-2.3**	**-1.6**	**-3.8**	**-3.6**	0.9	**-4.9**	**-1.9**	1.3	-1.6	-0.6	2.0	**-4.0**	**-4.1**	**-2.0**

[a]PMB, polymyxin B (2 μg/ml); RIF, rifampin (5 μg/ml); AMI, amikacin (20 μg/ml); MIN, minocycline (4 μg/ml); MER, meropenem (50 μg/ml). A green background indicates synergy (a $\geq$2-log$_{10}$ decrease in the number of CFU/ml with combination therapy compared with its most active component at the specified time and with the number of surviving bacteria in the presence of the combination being $\geq$2 log$_{10}$ CFU/ml below the starting inoculum); a red background indicates additivity (a $\geq$1.0-log$_{10}$ decrease in number of CFU/ml with the combination compared with its most active component, without being synergistic).

killing than the most active monotherapy) or additive against all isolates, including at 24 h in the majority of cases (Table 2 and Fig. S3). However, only the polymyxin B-rifampin-amikacin combination was additive or synergistic against all isolates at 24 h. Despite the observed additivity/synergy at 24 h, in most cases bacterial growth was trending upwards at this time.

One-compartment IVM. The most active triple combination from the static-concentration time-kill experiments, namely, polymyxin B-rifampin-amikacin, was further examined in an *in vitro* dynamic infection model (IVM) against isolates ATH 16 and BD 32. The results of IVM experiments are shown in Fig. 1 (time-kill curves) and 2 (population analysis profiles) and Table S1 (log changes in viable cell counts). The growth control grew steadily to ~8.5 log$_{10}$ CFU/ml throughout the experiment. Against

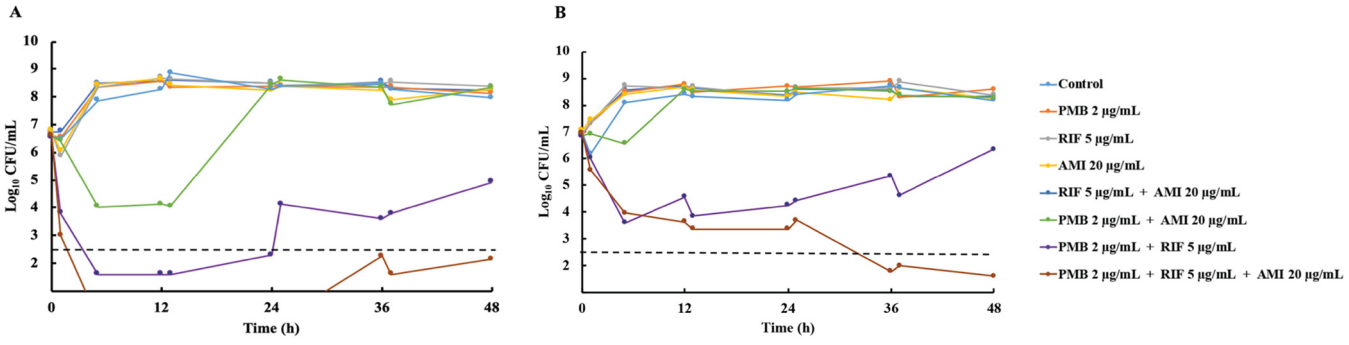

FIG 1 Time-kill curves with clinically relevant concentrations of polymyxin B (constant concentration of 2 μg/ml), rifampin (C_{max}, 5 μg/ml; $t_{1/2}$, 2.5 h), and amikacin (C_{max}, 20 μg/ml; $t_{1/2}$, 2.5 h) alone, in double combinations, and in a triple combination with an inoculum of ~10^7 CFU/ml against two isolates of polymyxin-resistant, MDR *K. pneumoniae* (ATH 16 [A] and BD 32 [B]) using a dynamic one-compartment infection model. The *y* axis starts from the limit of detection (~1.3 log$_{10}$ CFU/ml), and the dashed horizontal line represents the limit of quantification. PMB, polymyxin B; RIF, rifampin; AMI, amikacin.

ATH 16, monotherapy and the double combination of rifampin plus amikacin were ineffective, with growth mirroring that of the growth control. The combination of polymyxin B plus amikacin produced a maximum killing of ~3 $\log_{10}$ CFU/ml at 5 h, but regrowth to control values occurred by 24 h. The combination of polymyxin B plus rifampin produced maximal killing of ~5 $\log_{10}$ CFU/ml at 5 h, with slow regrowth (to ~5 $\log_{10}$ CFU/ml at 48 h) occurring thereafter. The polymyxin B-rifampin-amikacin triple combination was the most effective, with no viable bacteria detected from 5 to 25 h and regrowth to only ~2 $\log_{10}$ CFU/ml at 48 h. For the latter two combinations (polymyxin B-rifampin double and polymyxin B-rifampin-amikacin triple combinations), compared to the level of the most active monotherapy, additivity or synergy was observed at all times across 48 h (Table S1); on many occasions and including at 48 h, the triple combination was synergistic compared to all mono- and double-combination therapies. Against BD 32, only polymyxin B-rifampin and the triple combination showed substantial bacterial killing. With both combinations against BD 32, killing of ~3 $\log_{10}$ CFU/ml had occurred at 5 h; however, slow regrowth to ~6.5 $\log_{10}$ CFU/ml at 48 h occurred with polymyxin B-rifampin. Bacterial numbers continued to decline slowly with the triple combination, with growth of ~2 $\log_{10}$ CFU/ml at 48 h. With these two combinations, additivity and synergy patterns were similar to those observed with ATH 16.

Baseline population analysis profiles (PAPs) for both isolates showed that the entire population was highly resistant to polymyxin B (growing in the presence of 32 μg/ml polymyxin B) (Fig. 2). Subsequent PAPs at 24 and 48 h revealed that irrespective of the treatment regimen (polymyxin B monotherapy or polymyxin B double or triple therapy), virtually all bacteria detected remained highly resistant to polymyxin B.

Mouse thigh infection model. Log_{10} CFU/thigh counts in the mouse thigh infection model are shown in Fig. 3 and 4. In the control mice, values ranged from ~9 to 10 $\log_{10}$ CFU/thigh at 24 h. With polymyxin B monotherapy, little or no reduction in $\log_{10}$ CFU/thigh compared to that of the controls occurred at 24 h with any of the 6 isolates. Larger reductions (4 to 4.6 $\log_{10}$ CFU/thigh) occurred with the triple therapy, with a $\log_{10}$ CFU/thigh of ~5 observed at 24 h for all isolates; the latter value was at least 2.5 $\log_{10}$ CFU/thigh below that observed with polymyxin B monotherapy at this time and ~1 $\log_{10}$ CFU/thigh lower than that of the starting inoculum (~10^7 $\log_{10}$ CFU/thigh). Viable bacterial counts with triple therapy were significantly different from those of the control and polymyxin B monotherapy groups at 24 h, while no significant differences were observed between the polymyxin B monotherapy and untreated control groups at this time ($P < 0.05$). The *in vivo* efficacy of all treatments (polymyxin B, rifampin, or amikacin alone and in double and triple combinations) was further evaluated against BD 32 (Fig. 4). For all monotherapies, there was only a small reduction in bacterial counts at 24 h compared to the level for the control. Similarly, there was no improvement in bacterial killing at this time with the rifampin-amikacin combination, although larger reductions were observed with the polymyxin B-amikacin, polymyxin B-rifampin, and polymyxin B-rifampin-amikacin combinations. Only the polymyxin B-rifampin and triple combinations reduced the bacterial burden to below that of the initial inoculum.

DISCUSSION

As MDR *K. pneumoniae* spreads globally (26, 27), reports of strains resistant to the last-line polymyxins have become increasingly more frequent (22, 23). Such a dire situation leaves clinicians with virtually no therapeutic options to treat this problematic human pathogen. Polymyxin combination therapy is considered a viable clinical strategy to salvage bacterial killing efficacy against pathogens resistant to the individual monotherapies (28, 29). *In vitro* and *in vivo* studies provide considerable support for polymyxin use as part of combination therapies, including when combined with antibiotics (e.g., rifampin) that typically are inactive against Gram-negative organisms (29). Although polymyxin-based combinations are increasingly used clinically (30, 31), the choice of antimicrobial agents in these combinations often are based on trial and error or anecdotal experiences. Thus, investigating rational, scientifically based

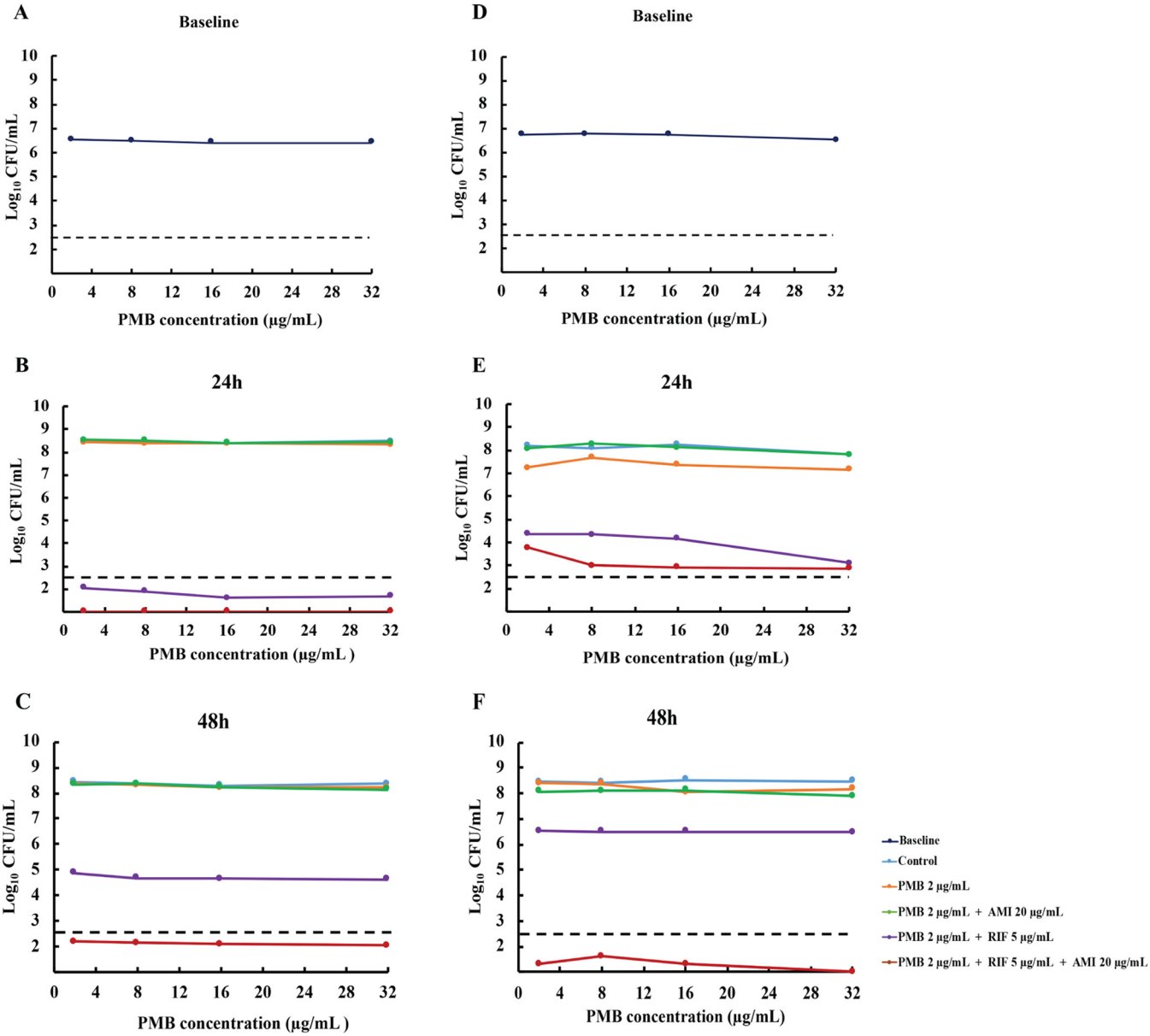

FIG 2 Population analysis profiles of polymyxin B at baseline (0 h; inoculum of ~10^7 CFU/ml) or 24 h and 48 h after exposure to no antibiotics (control) or clinically relevant concentrations of polymyxin B (constant concentration of 2 μg/ml) alone or combined with rifampin (C_{max}, 5 μg/ml; $t_{1/2}$, 2.5 h), and/or amikacin (C_{max}, 20 μg/ml; $t_{1/2}$, 2.5 h). (A to C) ATH 16. (D to F) BD 32. The *y* axis starts from the limit of detection (~1.3 $\log_{10}$ CFU/ml), and the dashed horizontal line represents the limit of quantification. PMB, polymyxin B; RIF, rifampin; AMI, amikacin.

polymyxin-based combinations is critical for the optimization of antimicrobial therapy. Our study systematically examined polymyxin combination therapy with other antibiotics (rifampin, amikacin, minocycline, and meropenem) against six polymyxin-resistant, MDR clinical isolates of *K. pneumoniae*. The time-kill studies revealed good initial killing (~3 to 7 $\log_{10}$ CFU/ml) against many of the isolates with the various combinations, although slightly greater initial bacterial killing and suppression of regrowth was observed with the triple combinations (polymyxin B-rifampin-amikacin) compared to that of the double combinations. Nevertheless, substantial regrowth occurred in most cases with these static-concentration experiments.

The antibiotics forming the most active triple combination from the static time-kill experiments (polymyxin B-rifampin-amikacin) subsequently were evaluated in the one-compartment pharmacokinetic/pharmacodynamic (PK/PD) model; notably, both *K.*

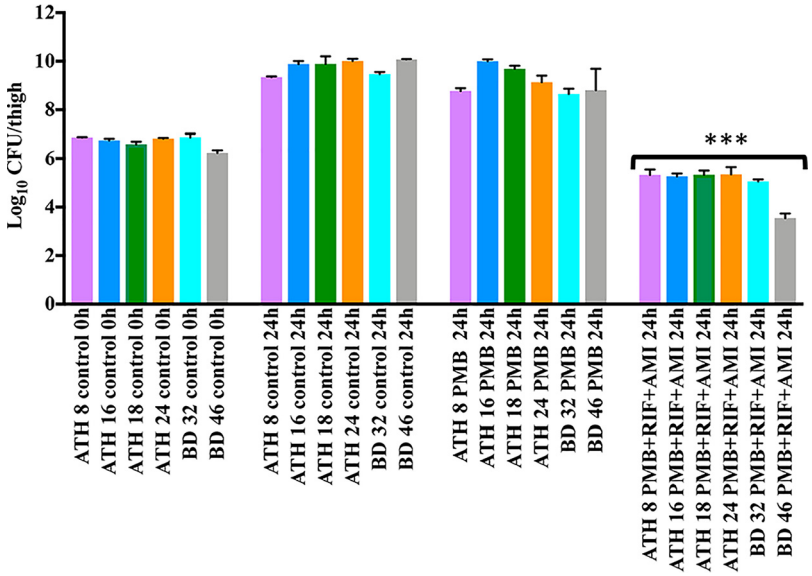

FIG 3 Log_{10} CFU/thigh counts in the mouse thigh infection model at baseline (0 h; inoculum of $\sim 10^7$ CFU/thigh) and 24 h after exposure to no antibiotics (control) or polymyxin B (60 mg/kg/day) alone or combined with rifampin (120 mg/kg/day) and amikacin (300 mg/kg/day) against six isolates of multidrug-resistant (including polymyxin-resistant) *K. pneumoniae*. PMB, polymyxin B; RIF, rifampin; AMI, amikacin. ***, Approximately 5 log_{10} CFU/thigh lower than that of the control group at 24 h ($P < 0.05$).

pneumoniae isolates tested in this experiment were resistant to each antibiotic in this combination when used as monotherapy. Our group has previously shown the importance of using dynamic models that mimic the antibiotic concentration-time profiles in patients when assessing the efficacy of antibiotic combinations (32, 33). The simulated plasma concentration-time profiles reflected free (unbound) concentrations achieved in patients using standard dosage regimens: polymyxin B at 2.5 mg/kg/day, giving an unbound average steady-state concentration ($fC_{ss,avg}$) of up to approximately 2 μg/ml (34, 35); rifampin at 600 mg every 12 h to achieve a peak unbound concentration (fC_{max}) of 5 μg/ml and half-life ($t_{1/2}$) of 2.5 h (36, 37); and amikacin at 7.5 mg/kg/12 h to achieve an fC_{max} of 20 μg/ml and $t_{1/2}$ of 2.5 h (38). The combination of polymyxin B plus rifampin and the triple combination were most effective against both extremely drug-resistant isolates tested, each causing substantial initial killing (>3 log_{10} CFU/ml),

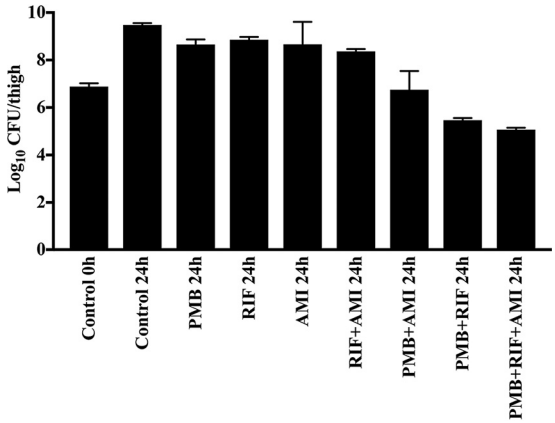

FIG 4 Log_{10} CFU/thigh counts in the mouse thigh infection model against polymyxin-resistant, MDR *K. pneumoniae* BD 32 at baseline (0 h; inoculum of $\sim 10^7$ CFU/thigh) and 24 h after exposure to no antibiotics (control) or polymyxin B (60 mg/kg/day), rifampin (120 mg/kg/day), and amikacin (300 mg/kg/day) alone, in double combinations, and in the triple combination ($n = 4$). PMB, polymyxin B; RIF, rifampin; AMI, amikacin.

and, for the triple therapy, continued killing across 48 h (BD 32) or with no detectable viable bacteria for a prolonged period, followed by minimal regrowth (ATH 16).

While a number of studies have examined polymyxin-based combinations against *K. pneumoniae*, very few have utilized polymyxin-resistant KPC-producing isolates (39–42). Using checkerboard and time-kill studies, Tascini et al. (43) and Gaibani et al. (44) found the combination of colistin plus rifampin was consistently synergistic against colistin-resistant, KPC-producing isolates of *K. pneumoniae*. Similarly, our results support the effectiveness of colistin/rifampin double combinations on initial bacterial killing (across the first 4 to 8 h). However, our static concentration and IVM studies with this double combination (the latter utilizing dynamic concentrations over 48 h; inoculum, $\sim 10^7$ CFU/ml) indicated that substantial regrowth occurs following the initial killing phase (Fig. 1; also see Fig. S2 in the supplemental material). Jernigan et al. (40) used time-kill studies (24 h, inoculum of 1×10^6 CFU/ml) to examine colistin combined with either doripenem, gentamicin, or doxycycline against 12 KPC-producing isolates of *K. pneumoniae* (10 were polymyxin-resistant isolates). Synergy at 24 h was observed with six isolates (colistin-doripenem), three isolates (colistin-gentamicin), and one isolate (colistin-doxycycline). In our time-kill study, the analogous combinations containing a polymyxin (polymyxin B) and a carbapenem (meropenem) or aminoglycoside (amikacin) were even less effective, with no synergy observed for any *K. pneumoniae* isolate at 24 h; apart from initial killing against ATH 16, the latter combination was similarly ineffective in the IVM.

Diep et al. (25) used an *in vitro* dynamic model (48 h, inoculum of $\sim 10^7$ CFU/ml) to examine the effectiveness of various triple combinations containing polymyxin B (constant concentration of 0.5, 1, or 2 μg/ml), rifampin (C_{max}, 5 μg/ml; $t_{1/2}$, 2 h; dosing every 8 and 12 h), and meropenem (C_{max}, 40 or 80 μg/ml; $t_{1/2}$, 2 h; dosing every 8 h) against one polymyxin-susceptible and one polymyxin-resistant KPC-producing *K. pneumoniae* isolate; both isolates were resistant to meropenem and rifampin. Irrespective of the individual regimen used for each antibiotic, the triple combination produced substantial bacterial killing against each isolate, with bacterial counts at 48 h at least ~ 3 to 4 $\log_{10}$ CFU/ml lower than that of the starting inoculum. In our static time-kill experiments, the same triple combination similarly enhanced bacterial killing, although the combination of polymyxin B-rifampin-amikacin was slightly more effective across all six isolates. To the best of our knowledge, ours is the first study to examine the triple combination of polymyxin B, rifampin, and amikacin in an IVM or *in vivo* against polymyxin-resistant, KPC-producing *K. pneumoniae*. The triple combination of polymyxin B, rifampin, and amikacin substantially enhanced bacterial killing in the IVM compared to the double combinations and was superior to polymyxin B monotherapy in the thigh infection model. Given a lack of therapeutic options and the absence of evidence-based treatment guidelines for treating patients with infections caused by KPC-producing *K. pneumoniae*, combination therapy with three or more antibiotics is increasingly used (40). In these patients, triple-combination therapies, of which a polymyxin is considered the backbone antibiotic, have been shown to significantly reduce overall mortality compared to monotherapy (45, 46).

The enhanced bacterial killing observed in this study with both double- and, especially, triple-combination therapy (polymyxin B-rifampin-amikacin) may be due to mechanistic synergy, whereby two drugs that act on different cellular pathways increase the rate and extent of killing of the other drug(s) (47). Although the exact mechanism(s) by which polymyxins exert their bactericidal action against Gram-negative bacteria remains unknown, electrostatically binding to lipopolysaccharide and permeabilization of the outer membrane, including against polymyxin-resistant strains (48, 49), is thought to be a major contributor. Such an action potentially enables the coadministered antibiotics to achieve greater access to their intracellular target sites, thereby enhancing activity. Bacterial killing by aminoglycosides is due primarily to the inhibition of protein synthesis via interaction with the 30S bacterial ribosome (50, 51). However, aminoglycosides, such as amikacin, at clinically relevant concentrations are known to exert an outer membrane permeabilizing effect (50, 52). This occurs prior to

any of their effects on protein synthesis and, thus, may increase the access of other antibacterial agents to their intracellular target sites. Rifampin exerts its action on bacteria by inhibiting bacterial DNA-dependent RNA polymerase (53, 54). Ordinarily, rifampin is inactive against Gram-negative bacteria, as this hydrophobic antibiotic is excluded from its target site by the outer membrane (54). Thus, the permeabilizing effect of both polymyxin B and amikacin may have facilitated the access of rifampin into cells. That said, the amikacin-rifampin combination performed poorly compared to the polymyxin B-rifampin combination in both static and dynamic *in vitro* models, suggesting polymyxin B facilitated greater entry than did amikacin. Thus, for the polymyxin B-rifampin-amikacin triple combination, the greatly enhanced bacterial killing observed may be due to the disruption of the outer membrane, primarily by polymyxin B, that facilitated the entry of both amikacin and rifampin to their intracellular targets. Although all isolates were resistant to amikacin, the increased intracellular amikacin concentrations might be sufficient to enhance the killing effect. Furthermore, our recent metabolomic study with isolate ATH 16 showed that the triple combination of polymyxin B-rifampin-amikacin negatively affects the lipid A modification pathway, thereby attenuating bacterial resistance to polymyxins (55). In addition, subpopulation synergy, namely, where one drug kills the resistant subpopulations of another drug and *vice versa* (47), seems unlikely to have significantly contributed to enhanced killing of the triple combination, given that all isolates were resistant (and likely remained resistant) to each drug.

Although antibiotic combination therapy can lead to improved clinical outcomes in patients infected with MDR isolates compared to monotherapy (56), a potential disadvantage of combination therapy is a greater risk of drug toxicity, including nephrotoxicity (57). Polymyxin B and amikacin monotherapy have dose-limiting nephrotoxicity (17, 58–60), while rifampin can cause hepatotoxicity (61). The average steady-state concentration ($C_{ss,avg}$) associated with mild nephrotoxicity for polymyxin B was estimated at ~4 μg/ml (34, 62), whereas for amikacin peak plasma concentrations (fC_{max}) greater than 40 μg/ml and trough levels (fC_{min}) above 10 μg/ml are associated with nephrotoxicity (63, 64). Despite the substantial improvements in *in vitro* and *in vivo* bacterial killing with the triple combination of polymyxin B-rifampin-amikacin, the potential of combining nephrotoxic agents might cause clinicians concern. Our studies utilized plasma concentrations of amikacin (fC_{max} of 20 μg/ml and minimum concentration [fC_{min}] of 1.02 μg/ml) that in patients would expose the kidneys to concentrations lower than those typically associated with nephrotoxicity. Additionally, inadequately treated Gram-negative infections can lead to sepsis and subsequent kidney injury (65). Thus, greater bacterial killing by the combination may allow for earlier recovery from sepsis and reduced kidney dysfunction.

Conclusions. The dissemination of MDR *K. pneumoniae* has created significant health care challenges worldwide and led to growing interest in optimizing antibiotic combination therapies to treat these organisms. We have demonstrated, for the first time, in a one-compartment PK/PD model and *in vivo* that a polymyxin-based triple combination with rifampin and amikacin significantly enhances bacterial killing against polymyxin-resistant, KPC-producing *K. pneumoniae*. Future PK/PD modeling and clinical trials are required to optimize dosing of this triple combination against MDR *K. pneumoniae*.

MATERIALS AND METHODS

Bacterial isolates, antibiotics, MICs, and media. Six isolates of *K. pneumoniae* were investigated. Four isolates (ATH 8, ATH 16, ATH 18, and ATH 24) were obtained from the Hygeia General Hospital, Marousi, Greece (2016), and two (BD 32 and BD 46) from SUNY Downstate Medical Center, Brooklyn, New York. All isolates were KPC-producing and multidrug resistant, i.e., nonsusceptible to at least one agent in three or more antimicrobial categories, which, in this case, included the polymyxins (66, 67). MICs to all tested antibiotics were determined in duplicate on separate days in cation-adjusted Muller-Hinton broth (CaMHB; Mg^{2+} at 12.2 μg/ml and Ca^{2+} at 23.0 μg/ml [Oxoid, Hampshire, UK]). The MIC breakpoint for minocycline was assigned according to the Clinical and Laboratory Standards Institute guidelines (CLSI) (68), and those of the other drugs (except polymyxin B) were assigned according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (69). Although no breakpoints for polymyxin

B against the *Enterobacteriaceae* have been established, susceptibility and resistance breakpoints for colistin have been set at ≤2 μg/ml and >2 μg/ml, respectively (69). For this study, we applied the colistin breakpoint to polymyxin B given the comparable activity of each polymyxin (70). The same medium was used for static-concentration and *in vitro* dynamic infection model experiments (discussed below).

Polymyxin B (PMB; lot number 20120204; Sigma-Aldrich, Castle Hill, Australia), amikacin (AMI; lot number 058K0803; Sigma-Aldrich), rifampin (RIF; lot number SLBK5059V; Sigma-Aldrich), minocycline (MIN; lot number 20120424; Sigma-Aldrich), and meropenem (MER; Fresenius Kabi, Mount Kuring-Gai, Australia) were carefully chosen from five major classes of antibiotics. Concentrations were selected to mimic the steady-state average concentrations ($fC_{ss,avg}$) or peak concentrations (fC_{max}) of free (unbound) drug in human plasma in patients administered standard-dosage regimens. In brief, sterile stock solutions of PMB, AMI, MER, and MIN were freshly prepared in Milli-Q water immediately prior to each experiment. RIF stock solutions were prepared in a minimum amount of dimethyl sulfoxide (DMSO) before dilution with Milli-Q water. All drug solutions were filter sterilized using 0.22-μm filters (Millipore, Bedford, MA).

Static-concentration time-kill experiments. Antibiotics were examined as monotherapy and in double and polymyxin B-containing triple combinations against six isolates (Table 2). Prior to each experiment, isolates were subcultured onto nutrient agar (Media Preparation Unit, Monash University, Clayton, Australia) and incubated at 37°C for 24 h. Single colonies were selected and grown overnight in 10 ml of CaMHB with constant shaking (180 rpm), from which early-log-phase bacterial cultures (~10^7 CFU/ml) were obtained (20 ml). Antibiotic solutions were added to achieve the desired drug concentrations (PMB, 2 μg/ml; RIF, 5 μg/ml; AMI, 20 μg/ml; MIN, 4 μg/ml; and MER, 50 μg/ml), whereupon tubes were incubated at 37°C in a shaking water bath (180 rpm) for 24 h. Serial samples (0.2 ml) were collected aseptically at 0, 1, 4, and 24 h, with viable counting conducted immediately via serial dilution (using 0.9% saline) and spiral plating (WASP2 spiral plater; Don Whitley Scientific, Ltd., UK) of 50 μl of undiluted or appropriately diluted suspension onto Mueller-Hinton II agar, followed by incubation at 37°C.

IVM. A one-compartment *in vitro* PK/PD model (33) was used to examine bacterial killing and resistance suppression over 48 h against isolates ATH 16 and BD 32. Polymyxin B, rifampin, or amikacin was used as a monotherapy and in double combinations (polymyxin B plus rifampin, polymyxin B plus amikacin, and rifampin plus amikacin) and the triple combination. Single colonies of each isolate were selected and grown overnight as described for static time-kill studies. An early-log-phase bacterial suspension then was prepared by the addition of 200 μl of the overnight culture to 20 ml of CaMHB, with 0.8 ml then inoculated into each reservoir of the model (containing 80 ml of CaMHB) to obtain the desired starting inoculum of ~10^7 CFU/ml. The temperature of each reservoir was maintained at 37°C via heating in paraffin oil and with constant stirring via a magnetic stirrer located within each reservoir.

Eight sealed reservoirs (compartments) were used, with one acting as a growth control to define growth dynamics in the absence of antibiotic. As polymyxin B was administered at a constant concentration, it was spiked into one central reservoir connected only to polymyxin B-containing reservoirs immediately prior to starting the experiment, such that all media flowing through these reservoirs contained polymyxin B at a constant concentration of 2 μg/ml; this simulated the upper limit of unbound average steady-state concentration ($fC_{ss,avg}$) observed in critically ill patients (35), with the steady-state concentration present from the beginning of each experiment. Following bacterial inoculation, rifampin and amikacin were administered as 1-h infusions every 12 h using an automatic syringe pump to attain unbound peak concentrations (fC_{max}) of 5 μg/ml and 20 μg/ml, respectively. Medium was pumped through each reservoir at a rate of 0.37 ml/min to simulate a plasma elimination half-life ($t_{1/2}$) of 2.5 h for both rifampin and amikacin; the chosen C_{max} and $t_{1/2}$ approximate those achieved in patients administered standard doses intravenously (36, 38, 71, 72).

Serial samples (0.5 ml) were collected from each reservoir aseptically at 0, 5, 12, 13, 24, 25, 36, 37, and 48 h for viable cell counting and at 24 and 48 h for population analysis profiles (PAPs). Viable counting was conducted as described for static time-kills but with an additional washing step to reduce the possibility of antibiotic carryover. Washing involved the centrifugation of the samples (10,000 × *g*, 4°C, 10 min) with resuspension in 0.9% saline prior to dilution. To evaluate the emergence of polymyxin B resistance, PAPs were performed. Similarly treated samples were plated onto Mueller-Hinton II agar containing polymyxin B at 2, 8, 16, or 32 μg/ml, followed by incubation at 37°C for 24 h. All colonies were counted manually.

Mouse thigh infection model. A previously described neutropenic mouse thigh infection model was used to evaluate the efficacy of the triple combination across 24 h (73). Mice were rendered neutropenic by intraperitoneal injection of two doses of cyclophosphamide (Endoxan; Baxter Healthcare Pty. Ltd., New South Wales, Australia) administered 4 days (150 mg/kg of body weight) and 1 day (100 mg/kg) prior to experimental infection. Mice were anesthetized briefly with isoflurane by inhalation prior to inoculation. An early logarithmic-phase bacterial suspension of 50 μl (~10^6 CFU/ml) was injected intramuscularly into each posterior thigh muscle, with antibiotic treatment commencing 2 h later. At this time, infection was reproducibly established with a starting mean bacteria burden for the six strains of ~10^7 CFU per thigh. Experiments were conducted using all six isolates.

Two control mice were sacrificed 2 h postinoculation ($t = 0$ h) to examine bacterial counts prior to antibiotic treatment. Polymyxin B (60 mg/kg/day), rifampin (120 mg/kg/day), and amikacin (300 mg/kg/day) each was examined as monotherapy and in double and triple combinations against isolate BD 32. Against the remaining five isolates, polymyxin alone and the triple combination were tested at equivalent concentrations. The antibiotic treatments then were commenced via subcutaneous injection every 8 h. The polymyxin B dose was chosen to mimic the exposure of unbound polymyxin B in human plasma based on animal scaling and its different plasma protein binding in humans and mice (35, 74). The doses

of rifampin and amikacin were determined based on the literature and consideration of toxicities (36, 38, 71, 72). Each dosage regimen and the remaining controls involved two mice and provided four data points. The pharmacodynamics of control mice (to determine the overall level of bacterial growth in the absence of treatment) and mice receiving treatment were determined at 24 h following humane killing of the mice. Both posterior thigh muscles were aseptically collected, homogenized in sterile saline at 9,000 × *g* for 30 s (T25 ULTRA-TURRAX; IKA), and filtered using a sterile filter bag (9.5 by 16 cm) with a pore size of 280 μm (Labtek Pty. Ltd.). After this, 1 ml of the filtrate was serially diluted for plating (usually 0.1 ml homogenate in 0.9 ml saline) on nutrient agar as previously described, followed by incubation at 37°C for 24 h. Colonies were counted using an automated colony counter (ProtoCol) and expressed as $\log_{10}$ CFU/thigh.

Pharmacodynamic analysis. Microbial responses were evaluated using the log change method quantified as follows: log change = $\log_{10}(CFU_t) - \log_{10}(CFU_0)$, where $\log_{10}(CFU_0)$ is the bacterial count at 0 h and $\log_{10}(CFU_t)$ the bacterial count at a specified time (*t*). For static and dynamic *in vitro* studies, synergy was considered to be a $\geq$2-$\log_{10}$ CFU/ml lower bacterial count with the combination compared to the most active single component at the specified time and with the number of surviving bacteria in the presence of the combination being $\geq$2 $\log_{10}$ CFU/ml below the starting inoculum (75). Additivity was considered a $\geq$1.0-$\log_{10}$ CFU/ml lower bacterial count at the specified time than that of the combination without being synergistic (75). Bactericidal activity was defined as a $\geq$3-$\log_{10}$ decrease in the number of CFU/ml compared to that of the starting inoculum. For the thigh infection studies, the viable bacterial counts between the control groups at 24 h and the treated groups at 24 h were statistically compared ($P < 0.05$) using Tukey's multiple-comparison test (GraphPad Prism 8 software; La Jolla, CA, USA).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

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We have no conflicts of interest to declare.

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