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Optimization of Polymyxin B in Combination with Doripenem To Combat Mutator *Pseudomonas aeruginosa*

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Development of spontaneous mutations in *Pseudomonas aeruginosa* has been associated with antibiotic failure, leading to high rates of morbidity and mortality. Our objective was to evaluate the pharmacodynamics of polymyxin B combinations against rapidly evolving *P. aeruginosa* mutator strains and to characterize the time course of bacterial killing and resistance via mechanism-based mathematical models. Polymyxin B or doripenem alone and in combination were evaluated against six *P. aeruginosa* strains: wild-type PAO1, mismatch repair (MMR)-deficient (*mutS* and *mutL*) strains, and 7,8-dihydro-8-oxo-deoxyguanosine system (GO) base excision repair (BER)-deficient (*mutM*, *mutT*, and *mutY*) strains over 48 h. Pharmacodynamic modeling was performed using S-ADAPT and facilitated by SADAPT-TRAN. Mutator strains displayed higher mutation frequencies than the wild type (>600-fold). Exposure to monotherapy was followed by regrowth, even at high polymyxin B concentrations of up to 16 mg/liter. Polymyxin B and doripenem combinations displayed enhanced killing activity against all strains where complete eradication was achieved for polymyxin B concentrations of >4 mg/liter and doripenem concentrations of 8 mg/liter. Modeling suggested that the proportion of preexisting polymyxin B-resistant subpopulations influenced the pharmacodynamic profiles for each strain uniquely (fraction of resistance values are −8.81 $\log_{10}$ for the wild type, −4.71 for the *mutS* mutant, and −7.40 $\log_{10}$ for the *mutM* mutant). Our findings provide insight into the optimization of polymyxin B and doripenem combinations against *P. aeruginosa* mutator strains.

The term "hypermutator" classically refers to bacterial strains that display a >100-fold increase in the rate of spontaneous mutations and are present in up to 57% of patient isolates in certain chronic infections, such as cystic fibrosis, which harbor *Pseudomonas aeruginosa* (1–3). During the course of chronic infection, the presence of the mutator phenotype was proposed to provide an added adaptive advantage in addition to those provided by normal spontaneous or DNA damage-induced mutations, for example, by increasing the acquisition of antibiotic resistance (4). Generally speaking, mutations in mutator strains result from a deficiency of one or more DNA repair mechanisms. Defects in the mismatch repair (MMR) system are responsible for up to 67% of all mutator phenotype strains identified in the environment (3). Strains impaired in the 7,8-dihydro-8-oxo-deoxyguanosine (GO) base excision repair (BER) system have also been described (1).

The MMR system (*mutS* and *mutL*) detects and repairs single-base mismatches or small insertions or deletions resulting from errors made during DNA replication (5, 6). Clinically, MMR deficiency is commonly observed in *P. aeruginosa* isolated from cystic fibrosis patients chronically colonized with this organism (1–3). It has been hypothesized that during chronic infection or at stationary phase, MMR is transiently shut down to increase mutation frequency and promote the selection of antibiotic-resistant clones (7). Accordingly, strains that possess the mutator phenotype are a therapeutic problem since they can rapidly develop resistance due to natural genomic error and lack of DNA repair mechanisms. However, we recently described how the emergence of polymyxin B resistance in *mutS P. aeruginosa* resulted in attenuation of virulence (8), underscoring the complex and poorly understood relationship between hypermutability and pathoadaptation (9).

The guanine BER system, a repair pathway involving the *mutT*, *mutM*, and *mutY* genes, is a critical system for repairing DNA damage induced by reactive oxygen species (ROS) in *P. aeruginosa* (10). Inflammation during infection exposes *P. aeruginosa* to high levels of ROS that induce DNA damage (10) and/or saturate DNA repair mechanisms. Although a number of studies have examined fundamental mutagenesis mechanisms of the MMR or oxidized guanine BER system, there is a paucity of data regarding antibiotic pharmacodynamics (PD) against mutator strains. This is particu-

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TABLE 1 Isogenic bacterial strains and corresponding polymyxin B and doripenem MICs

Strain	Relevant genotype (reference)	MIC (mg/liter)	
		Polymyxin B	Doripenem
Wild type	MPAO1: PAO1 wild-type strain (34)	1	0.5
mutS mutant	MPA32417 (MPAO1-*mutS*): MPAO1 bearing *mutS*::IS*phoA/hah* (loss-of-function *mutS* mutant) (34)	2	2
mutL mutant	MPAO1 (loss-of-function *mutL* mutant)	2	2
mutM mutant	MPA16280 (MPAO1-*mutM*): MPAO1 bearing *mutM*::IS*lacZ/hah* (loss-of-function *mutM* mutant) (10)	1	0.5
mutT mutant	MPA52426 (MPAO1-*mutT*): MPAO1 bearing *mutT*::IS*phoA/hah* (10)	1	0.5
mutY mutant	MPA39575 (MPAO1-*mutY*): MPAO1 bearing *mutY*::IS*phoA/hah* (loss-of-function *mutY* mutant) (10)	1	1

larly important as it relates to the increasing tide of Gram-negative resistance to clinically important antibiotics.

The use of the polymyxins, an "old" class of antibiotics, was abandoned due to an increased incidence of dose-limiting toxicities. Recently, clinicians have been forced to use polymyxins as a last resort against Gram-negative pathogens due to the lack of viable treatment options (11–14). Polymyxin monotherapy results in rapid initial killing of *P. aeruginosa*, followed by extensive regrowth of resistant subpopulations, which is particularly problematic at high bacterial densities (15–22). Therefore, polymyxin-based combination approaches may offer a viable alternative treatment option against these difficult-to-treat infections (16, 19, 23–27). Such a strategy may be even more useful against rapidly adapting strains, which harbor the mutator phenotype. Carbapenems, when combined with polymyxin B, have displayed potent *in vitro* synergy in addition to resistance suppression, and their combination has also led to improved clinical outcomes in critically ill patients with *P. aeruginosa* infections (28, 29). Furthermore, *P. aeruginosa* is frequently more susceptible to doripenem than imipenem or meropenem (30, 31) and has a low incidence for doripenem resistance acquisition, especially when used in combination (32, 33). Here, we evaluated the pharmacodynamics of polymyxin B and combinations with doripenem against *P. aeruginosa* wild-type and mutator strains to determine the potential utility of these combinations.

MATERIALS AND METHODS

Bacterial isolates, antibiotics, susceptibility tests, and mutation frequency. Wild-type and five isogenic strains with a deficiency in the MMR DNA repair system (34) or the GO BER system (10) were used in our studies. The genotypes and antibiotic MICs are listed in Table 1.

Polymyxin B (sulfate) and rifampin were obtained from Sigma Chemical Company (St. Louis, MO) and doripenem was kindly provided by Johnson & Johnson (New Brunswick, NJ). Stock solutions were prepared by dissolving polymyxin B in sterile distilled water and doripenem in 0.9% normal saline. Each of the stock solutions was sterile filtered using a 0.22-μm-pore-size Millex-GP filter (Millipore, Bedford, MA). All time-killing experiments were performed using Luria-Bertani (LB) broth supplemented with magnesium (12.5 mg/liter) and calcium (25 mg/liter). LB agar (Difco Laboratories, Detroit, MI) was used for culturing the PAO1 wild type for each experiment and for measuring the total viable cell counts. LB agar plates containing 60 mg of tetracycline/liter were used for culturing the mutant isolates containing tetracycline cassettes.

MICs were determined in quadruplicate according to Clinical and Laboratory Standards Institute guidelines (35). The mutation frequency was determined by plating an overnight bacterial culture on LB agar containing 100 mg of rifampin/liter (36, 37). The mutation frequency was defined as the proportion of bacterial cells that grew on drug containing agar divided by the total population.

Time-kill experiments. Time-kill experiments were performed as previously described (38). Briefly, overnight bacterial cultures were used to prepare the initial inoculum for each strain. The optical density of the bacterial suspension, measured at 620 nm, was adjusted to ~1 for subsequent dilution to the desired initial inoculum. Initial time-kill experiments with polymyxin B alone were conducted at two different initial inocula (10^6 and 10^8 CFU/ml) to determine whether there was an inoculum effect for mutator strains and where combination exploration with doripenem would be most appropriate. Time-kill experiments using polymyxin B alone (0, 1, 2, 4, 8, 16, and 64 mg/liter) were performed for the six *P. aeruginosa* strains. Doripenem (2 and 8 mg/liter) was tested alone and in combination with polymyxin B (0, 2, 4, 8, and 16 mg/liter) against the wild-type, *mutS* mutant, and *mutM* mutant strains at 10^8 CFU/ml. Ten bacterial samples were serially collected at 0, 0.5, 1, 2, 4, 8, 24, 28, 32, and 48 h and plated on LB medium to quantify the viable bacterial density. The change in $\log_{10}$CFU/ml from baseline at 8, 24, and 48 h was calculated from the raw data to characterize the early- and late-killing activity of antibiotics alone and in combination.

Mechanism-based modeling. (i) Model development and structure. Mechanism-based PD models were developed to quantitatively characterize the bacterial killing and regrowth for both monotherapy and polymyxin B-based combination regimens with doripenem against wild-type and *mutS* and *mutM* mutator strains. Multiple models with different permutations of subpopulations (up to a maximum of 6 [i.e., 3×2] different subpopulations) with different susceptibilities to each of the antibiotics were evaluated. We considered models with and without interconversion between different subpopulations. These bacterial subpopulations included polymyxin B-susceptible (PB^s; MIC $\leq$ 2 μg/ml), polymyxin B-intermediate (PB^i; MIC = 4 μg/ml), and polymyxin B-resistant (PB^r; MIC $\geq$ 8 μg/ml) subpopulations combined with doripenem-susceptible (DOR^s; MIC $\leq$ 2 μg/ml) and doripenem-resistant (DOR^r) subpopulations, yielding six possible permutations. The final model was selected based on diagnostic plots, the objective function and plausibility of parameter estimates. The final model contained three subpopulations (PB^s/DOR^s, PB^i/DOR^s, and PB^r/DOR^s) for the wild-type and *mutM* strains and two subpopulations (PB^i/DOR^s and PB^r/DOR^s) for the *mutS* strain (see Fig. S1 in the supplemental material).

A life cycle growth model as previously described was used to model bacterial growth kinetics dividing each subpopulation into a vegetative and a replicating state (39, 40). Polymyxin B activity was characterized by target site binding, as previously described by Bulitta et al. (20). In brief, it was assumed polymyxin B is competitively displaced by Ca^{2+} and Mg^{2+} at the outer membrane of *P. aeruginosa*, which is the initial site of polymyxin action (41). Two types of synergy, namely, "subpopulation synergy" [i.e., polymyxin B killing the doripenem-resistant subpopulation(s) and vice versa] and "mechanistic synergy" were considered to explain the interaction between polymyxin B and doripenem (40). Mechanistic synergy was evaluated based on the assumption that polymyxin B could enhance killing by doripenem, that doripenem could enhance killing by polymyxin B, or both (see Fig. S1 in the supplemental material for a structural model and additional modeling detail). The doripenem *in vitro* degradation half-life was set at 50 h for our modeling, a value determined by fitting the

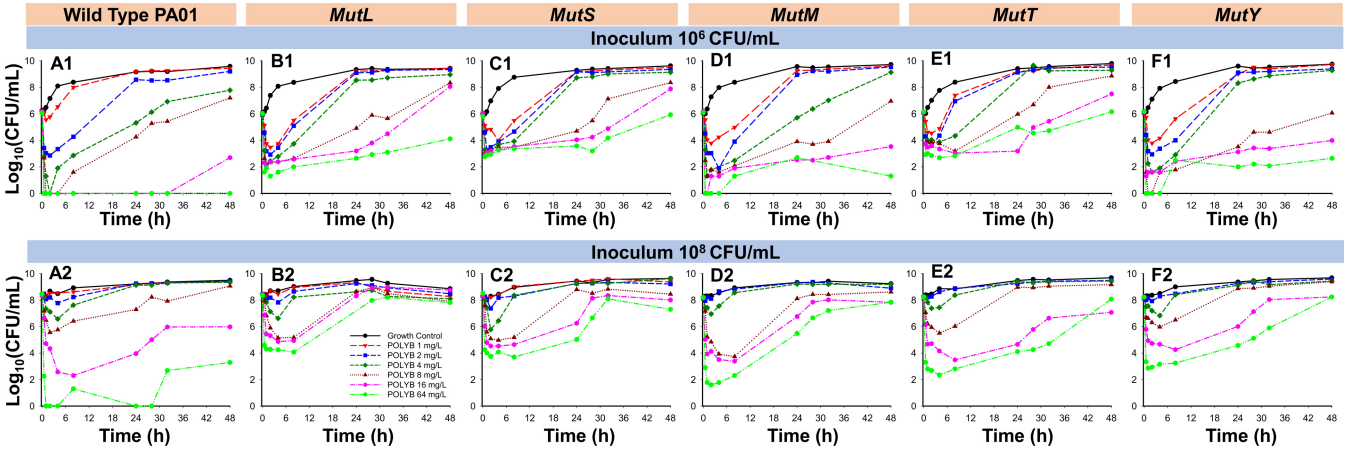

FIG 1 Time-kill experiments were performed comparing the pharmacodynamic activity of polymyxin B alone (1, 2, 4, 8, 16, and 64 mg/liter) against six strains of *P. aeruginosa*, including the wild-type strain (PAO1), isogenic MMR-deficient strains (*mutS* or *mutL*), and isogenic GO BER-deficient strains (*mutM*, *mutT*, or *mutY*), over 48 h at two different inocula (A1 to F1 and A2 to F2).

monoexponential decay model to the data obtained from Berthoin et al. (68). The polymyxin B concentration was assumed to be constant throughout the experiments since polymyxin B is known to be stable *in vitro* (42). The modeling methods used were described previously (20, 43–48).

(ii) Estimation. Candidate models were estimated by simultaneously fitting the viable count profiles using the importance sampling Monte Carlo parametric expectation maximization method (pmethod = 4) in the parallelized S-ADAPT software (version 1.57) facilitated by the SADAPT-TRAN pre- and postprocessing tool (46). An additive residual error variance model on $\log_{10}$ scale was used for bacterial counts of ≥100 CFU/ml, and an error model containing a Poisson error was used for bacterial counts that were <100 CFU/ml (20, 40).

PK and PD simulations. (i) PK models. Previously published human pharmacokinetic (PK) models for polymyxin B and doripenem were used as the driving function of our pharmacodynamic (PD) model. Polymyxin B PK data were described by a two-compartment model with linear clearance by Sandri et al. (49). The total body clearance of polymyxin B, when scaled by total body weight using a population mean, was 0.0276 liters/h/kg with a 32.4% interindividual coefficient of variation. The covariates that were previously found to significantly impact polymyxin B PK were not applied in the present study.

For doripenem, disposition in humans was described by a two-compartment model with linear clearance (50), including population mean parameter estimates and (% coefficient of variation) as follows: the total clearance (liters/h), the central volume of distribution (liters), the peripheral volume of distribution (liters), and the intercompartment distributional clearance (liters/h) were 13.6 (19%), 11.6 (19%), 6.0 (25%), and 4.7 (42%), respectively. The patient body weight was used as a covariate to describe the volume of distribution of the central compartment and the distributional clearance between compartments. The body weight, creatinine clearance, and age were used as covariates to describe the volume of distribution of the peripheral compartment. Creatinine clearance was used as a covariate for the total clearance from the central compartment.

(ii) Simulated dosage regimens. The following regimens for polymyxin B were simulated for 48 h *in silico*, based on the model by Sandri et al. (49): (i) traditional, 1.25 mg/kg every 12 h (q12h); (ii) traditional, 1.5 mg/kg q12h; (iii) front loaded, 2.0 mg/kg q12h × two doses, followed by 1.25 mg/kg q12h thereafter; (iv) front loaded, 2.0 mg/kg q12h × two doses, followed by 1.50 mg/kg q12h thereafter; (v) front loaded, 2.5 mg/kg q12h × two doses, followed by 1.50 mg/kg q12h thereafter; and (vi) "burst," 2.5, 5.0, 7.5, or 10 mg/kg q12h × two doses, followed by no additional doses thereafter. Doripenem was simulated *in silico* at 250, 500, 750, and 1,000 mg every 8 h, based on the recommended dosing in the package insert as monotherapy or in combination with polymyxin B dosed as above. Since renal function has a negligible effect on polymyxin B PK (43), only a single value for renal function (CL_{CR} = 28.7 ml/min/1.73 m^2) was simulated using Berkeley Madonna (v8.3.18). Simulations were based on the population mean PK and PD parameter estimates, and variabilities in the PK and PD parameters were not included.

RESULTS

Spontaneous mutation frequency. All mutator strains displayed a >600-fold increase in their spontaneous mutation frequency compared to the wild-type strain when grown on rifampin-containing agar.

Polymyxin B monotherapy. At low initial inocula (10^6 CFU/ml), polymyxin B (4 and 8 mg/liter) displayed killing levels against the *mutM* and *mutY* mutator strains similar to those against the wild-type strain (maximal bacterial reductions of 4.6, 4.1, and 4.3 $\log_{10}$ CFU/ml, respectively). Strains deficient in the MMR system (*mutL* and *mutS* mutants) and the BER-deficient *mutT* strain displayed 3.38, 2.57, and 2.39 $\log_{10}$ CFU/ml killing (Fig. 1). High-dose polymyxin B (>8 mg/liter) resulted in complete eradication of the wild type over 48 h, while the same polymyxin B exposure resulted in rapid initial killing during the first 0.5 h, followed by regrowth for the *mutM* and *mutY* strains. The same concentration against the *mutL*, *mutS*, and *mutT* strains resulted in bacterial reductions of ≤4.7 $\log_{10}$ CFU/ml within the first 4 h, followed by regrowth.

In contrast to the killing observed at low inocula, polymyxin B concentrations of ≤4 mg/liter displayed reduced activity at a high initial inoculum (10^8 CFU/ml), with maximal bacterial reductions of <0.82 $\log_{10}$ CFU/ml, for all strains. Upon exposure to a the higher polymyxin B concentration (8 mg/liter), the wild-type, *mutT*, and *mutY* strains displayed similar killing and regrowth profiles; however, the *mutS*, *mutL*, and *mutM* strains displayed greater initial killing (4.50 $\log_{10}$ CFU/ml), followed by regrowth comparable to the growth control (Fig. 1). For the highest concentration of polymyxin B (64 mg/liter) *mutS* and *mutL* strains displayed bacterial reductions of ≤4.8 $\log_{10}$ CFU/ml, followed by regrowth; the *mutM*, *mutT*, and *mutY* mutant strains displayed bacterial reductions of ≤6.6 $\log_{10}$ CFU/ml, followed by regrowth; and the wild-type strain displayed a maximal bacte-

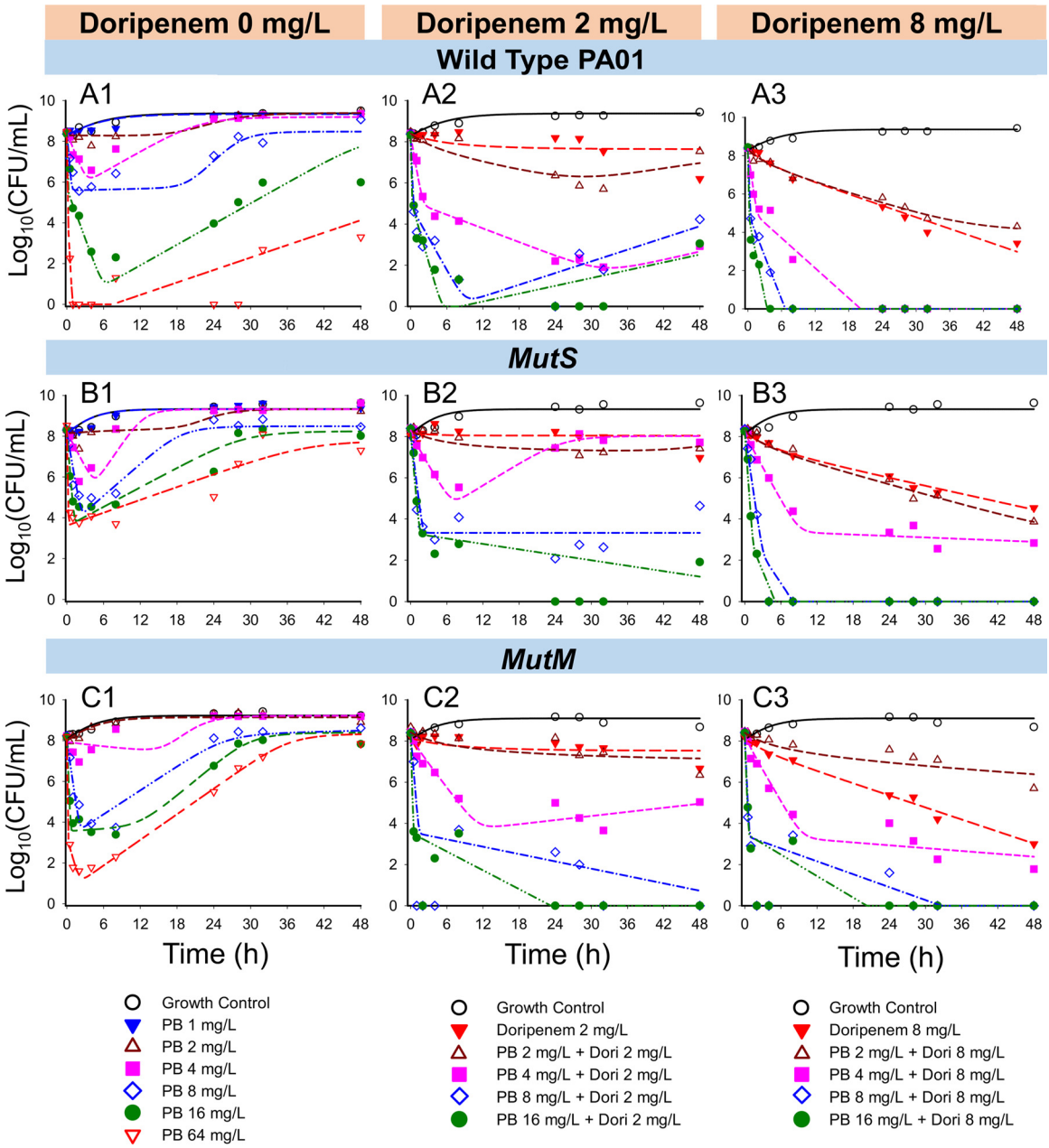

FIG 2 Time-kill experiments were performed to determine the activity of polymyxin B with doripenem in combination against wild-type PAO1 (A1 to A5) and mutator isogenic strains: *mutS* (B1 to B5) and *mutM* (E1 to E5) at an initial inoculum of 10^8 CFU/ml. (A1, B1, and C1) Polymyxin B alone; (A2, B2, and C2) polymyxin B combined with doripenem 2 mg/liter; (A3, B3, and C3) polymyxin B combined with doripenem 8 mg/liter. Data for polymyxin B and doripenem against all three strains were fitted by the proposed PD model.

rial reduction of 8.5 $\log_{10}$ CFU/ml before regrowth to 3.30 $\log_{10}$ CFU/ml (Fig. 1).

Doripenem monotherapy. Doripenem displayed similar killing profiles against the wild-type, *mutS*, and *mutM* strains (see Fig. S2A in the supplemental material). Doripenem at 2 mg/liter did not show bactericidal activity against any strain. The total bacterial reduction for all strains after exposure to doripenem at 8 mg/liter for 8, 24, and 48 h ranged from 1.12 to 1.61, 2.16 to 3.05, and 3.70 to 5.20 $\log_{10}$ CFU/ml, respectively.

Polymyxin B and doripenem combination therapy. Polymyxin B (2 mg/liter) in combination with doripenem (2 or 8 mg/liter) demonstrated rapid killing and ultimately maximal bacterial reductions of 4.1, 4.5, and 2.8 $\log_{10}$ CFU/ml for the wild-type, *mutS*, and *mutM* strains at 48 h (Fig. 2). Polymyxin B (4 mg/liter)-based doripenem combinations against the wild-type strain displayed rapid killing during the first 8 h and maximal bacterial reductions of 8.4 $\log_{10}$ CFU/ml after 48 h (Fig. 2A). Similarly, against the *mutM* strain, the maximum killing of this treatment combination was 6.7 $\log_{10}$ CFU/ml compared to 5.20 $\log_{10}$ CFU/ml for the most active antibiotic alone (Fig. 2C). Polymyxin B (4 mg/liter) and doripenem (2 mg/liter) against the *mutS* strain displayed a maximal initial bacterial reduction of 2.9 $\log_{10}$

TABLE 2 Parameter estimations of the mechanism-based models for polymyxin B and doripenem[a]

Parameter	Measurement (U)	Mean (%SE)		
		PA01 (wild type)	PA01 (Δ*mutS*)	PA01 (Δ*mutM*)
Maximum population size	Log_{10} CFU_{max}	9.41 (1.47)	9.32 (1.58)	9.21 (1.63)
Maximum population size with treatment	Log_{10} CFU_{max_T}	8.45 (1.93)	8.53 (1.29)	8.36 (1.71)
Initial inoculum	Log_{10} CFU_o	8.34 (1.03)	8.23 (0.858)	8.31 (1.44)
Initial inoculum for combination				8.11[b] (1.24)
Log_{10} (mutation frequencies)				
PB^i/DOR^s	Log_{10} FR_1	−3.07 (10.5)	–[c]	−4.64 (4.23)
PB^r/DOR^s	Log_{10} FR_2	−8.59 (4.8)	−4.43 (3.28)	−7.2 (3.94)
Doubling half-life	$t_{1/2}$ (min)	96.0 (7.27)	63.3 (8.29)	80.1 (8.51)
Fraction of receptors unoccupied by Ca^{2+} and Mg^{2+}, resulting in a polymyxin B concn at the outer membrane target site of 50% the polymyxin B concn in broth	EC_{50}[d]	0.731	0.618	0.825
Second-order killing rate constants for polymyxin B for:				
Susceptible population	k_{2S} [liters/(mg · h)]	2.46 (23.1)	–[c]	2.02 (13.1)
Intermediate population	k_{2I} [liters/(mg · h)]	0.193 (26.2)	0.568 (9.88)	0.0558 (15.6)
Resistant population for monotherapy	k_{2RM}[e] [liters/(mg · h)]	$0.351 \cdot 10^{-3}$ (50.1)	$7.11 \cdot 10^{-3}$ (59.6)	$0.435 \cdot 10^{-3}$ (15.3)
Resistant population for combination	k_{2RC}[e] [liters/(mg · h)]	$2.16 \cdot 10^{-3}$ (23.4)		0.236 (26.2)
Doripenem susceptible: concn needed to achieve 50% maximum killing by doripenem	KC_{50S} (mg/liter)	3.94 (27.8)	4.20 (17.7)	2.50[f]
Maximum killing rate constants for susceptible population	K_{max_S} (h^{-1})	0.954 (16.4)	0.979 (11.1)	0.783 (11.7)
Doripenem maximum killing rate altered by polymyxin B via a linear relationship	K_{STIM}[g] [liters/(mg · h)]		0.144 (25.3)	
SD of additive error on a log_{10} scale	SD_{CFU}	0.449 (6.23)	0.405 (5.98)	0.413 (6.14)

[a] The biological variability between-curve variability was set to a coefficient of variation of 15% for all parameters.
[b] The *mutM* strain initial inoculum was allowed to differ between monotherapy and combination treatment.
[c] –, the *mutS* strain was assumed to only have intermediate and resistant subpopulations, whereas the other two strains have three subpopulations.
[d] The 50% effective concentration (EC_{50}) was estimated via a logistic transform.
[e] The slower killing of the polymyxin B-resistant subpopulation was observed in the data; thus, we allowed for the different killing rate constants.
[f] The KC_{50} value for the *mutM* mutant was fixed at 2.5 mg/liter to improve the stability during estimation.
[g] Enhancement of killing by doripenem in the presence of polymyxin B was only part of the model for the *mutS* strain.

CFU/ml (Fig. 2B2), followed by regrowth. The wild-type and *mutM* strains displayed kill rates of up to 6.20 and 4.43 log_{10} CFU/ml, respectively (Fig. 2A2 and 2C2). Polymyxin B (≥8 mg/liter) and doripenem (2 mg/liter or 8 mg/liter) displayed up to 8.37 log_{10} CFU/ml of killing against all strains (Fig. 2).

Mechanism-based modeling. A mathematical model based on the known mechanisms of action of polymyxins and doripenem was developed to characterize the antibiotic effects against the wild-type PAO1 strain and the isogenic *mutM*- and *mutS*-deficient strains. Three subpopulations (PB^s/DOR^s, PB^i/DOR^s, and PB^r/DOR^s) were used to describe the time course of viable bacterial counts for the PAO1 wild-type and *mutM* mutant strains. Two subpopulations (PB^i/DOR^s and PB^r/DOR^s) were used to describe the *mutS* strain (see Fig. S1 in the supplemental material). The presence of the polymyxin B subpopulations was confirmed in each strain using population analysis profiles (data not shown). These models described well the viable bacterial count profiles for monotherapy and combination treatment (Fig. 2 and see Fig. S2 in the supplemental material) with correlation coefficients for the observed versus individual (population) fitted log_{10} viable counts of 0.979 (0.885) for the PAO1 wild-type strain, 0.979 (0.883) for the *mutS* strain, and 0.967 (0.944) for *mutM* strain (see Fig. S3 in the supplemental material).

For doripenem, the maximum killing rate constant (K_{max_S}) was 0.954 h^{-1} for the wild type, 0.979 h^{-1} for the *mutS* strain, and 0.783 h^{-1} for the *mutM* strain (Table 2). The doripenem concentration achieving half-maximal killing (KC_{50}) was comparable across strains. For the PAO1 wild-type and *mutM* strains, the PB^s/DOR^s subpopulation was rapidly killed by polymyxin B (Table 2), while the *mutS* strain lacked this subpopulation. The polymyxin B-resistant subpopulation displayed very slow killing rate constants [$k_{2RM} \leq 7.11 \cdot 10^{-3}$ liters/(mg · h)] for all strains against polymyxin B monotherapy (Table 2).

Subpopulation synergy was implemented as doripenem killing the PB^i and PB^r subpopulations for all strains but was not sufficient to describe the enhanced killing for the combinations. Thus, mechanistic synergy was used to help describe the additional benefit of combination treatment over monotherapy. For the wild-type and *mutM* strains, mechanistic synergy was best described by doripenem enhancing the rate of killing by polymyxin B against the polymyxin B resistant subpopulation. The latter population was killed 6.1-fold faster (k_{2RC}/k_{2RM}) by polymyxin B for the wild-

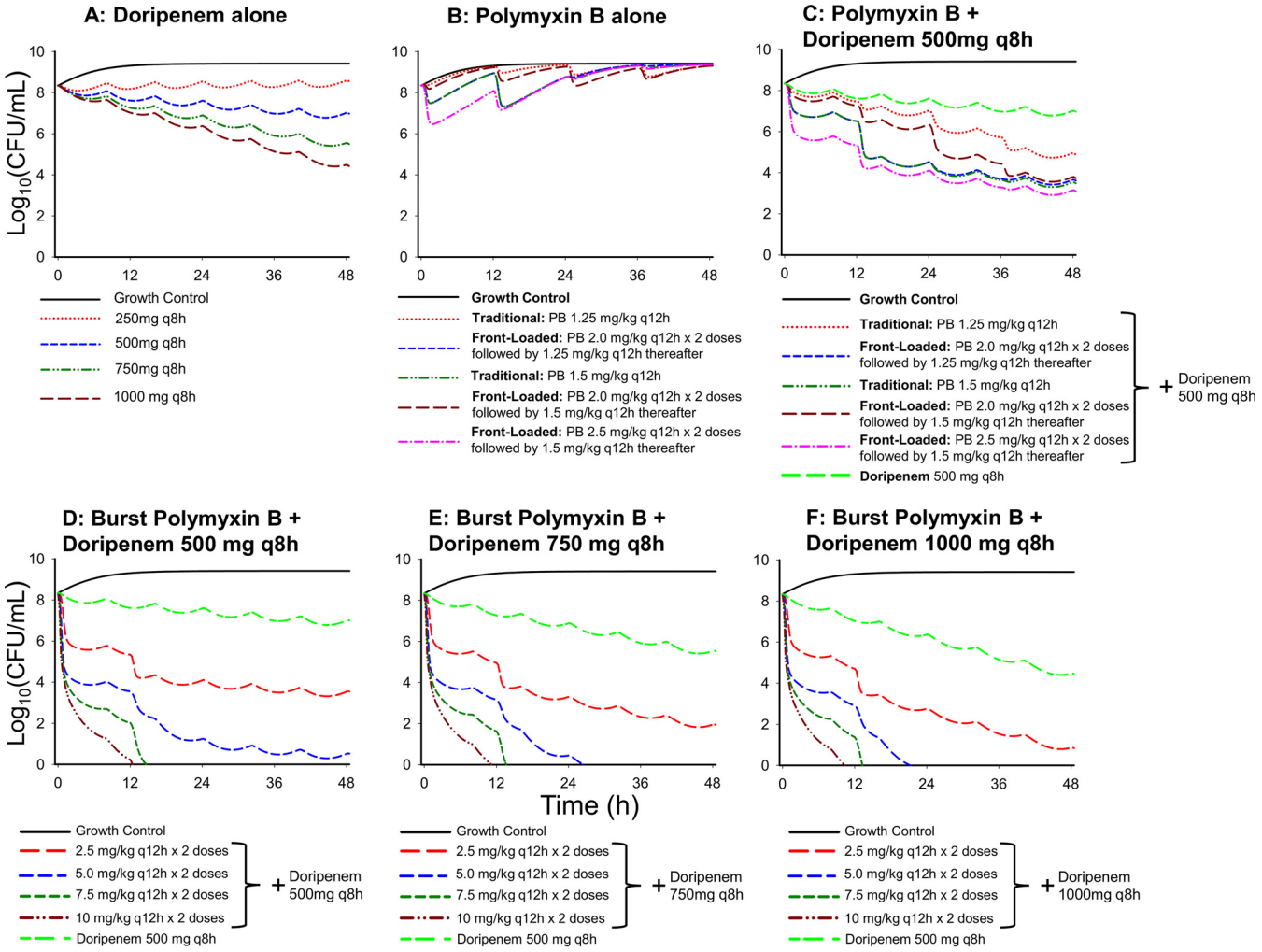

FIG 3 PK/PD simulation of doripenem and polymyxin B monotherapy and combination therapy against wild-type PAO1. Doripenem monotherapy (A), polymyxin B monotherapy (B), doripenem at 500 mg every 8 h in combination with various clinical doses of polymyxin B (C), doripenem at 500 mg every 8 h in combination with various polymyxin B "burst" doses (PB dosing only for 1 day) (D), doripenem at 750 mg every 8 h in combination with various polymyxin B burst doses (PB dosing only for 1 day) (E), and doripenem at 1,000 mg every 8 h in combination with various polymyxin B burst doses (PB dosing only for 1 day) (F) are shown.

type strain and 542-fold faster for the *mutM* strain. To describe mechanistic synergy for the *mutS* strain, polymyxin B was also assumed to enhance rate of killing by doripenem. Polymyxin B was modeled to linearly increase the maximum killing rate constant for doripenem (eq. S8). $K_{max\_S\_ALL}$ is the maximum killing rate constant of doripenem in combination therapy, and K_{max_S} is the maximum killing rate constant of doripenem for monotherapy. The slope (K_{STIM}) estimated for the polymyxin B concentration was 0.144 liters/(mg · h) (Table 2).

PK/PD simulations. Simulation results for the PAO1 wild-type strain are shown in Fig. 3. Doripenem monotherapy regimens displayed steady killing, where the highest dose of 1,000 mg administered every 8 h was predicted to result in 99.9% kill by 48 h. Polymyxin B monotherapy displayed minimal activity at the simulated doses. The simulated range of traditional or front-loaded polymyxin B regimens in combination with doripenem at 500 mg given every 8 h displayed earlier bactericidal killing than doripenem monotherapy (Fig. 3C).

For the wild-type strain, a polymyxin B burst regimen of ≥5 mg/kg administered q12h for 1 day in combination with 500 mg of doripenem given every 8 h resulted in rapid killing and suppression of regrowth (Fig. 3D). Eradication was predicted for a total polymyxin B dose on day 1 of ≥10 mg/kg/day in combination with 500 mg of doripenem every 8 h. A burst regimen of polymyxin B in combination with high-dose doripenem (i.e., 750 or 1,000 mg every 8 h) resulted in complete eradication, even for polymyxin B at 5 mg/kg/day (Fig. 3E and F).

Against the *mutS* strain, polymyxin B in combination with doripenem at 500 mg given every 8 h was predicted to yield substantial killing compared to monotherapy but was unable to eradicate the bacterial population (Fig. 4C). A burst regimen in combination with doripenem resulted in rapid killing for the high polymyxin B dose, followed by slight regrowth (Fig. 4D). The regrowth was suppressed at higher doripenem doses (Fig. 4E and F).

DISCUSSION

The development and selection of antibiotic resistance has been associated with the expedited evolutionary capacity of mutator strains (51). Infections caused by mutator strains are particularly difficult to treat given their high rate of spontaneous replication

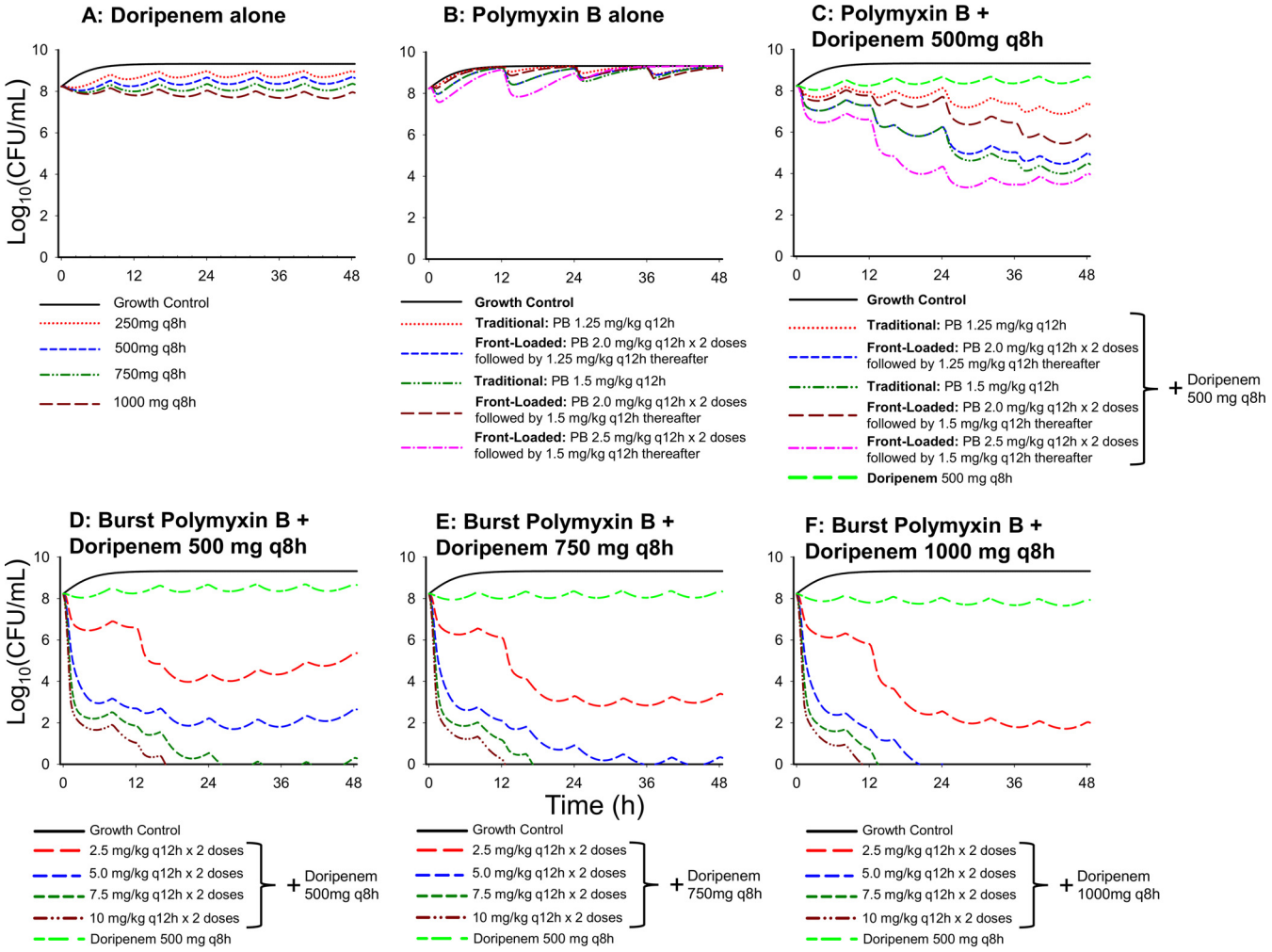

FIG 4 PK/PD simulation of doripenem and polymyxin B monotherapy and combination therapy against the *mutS* strain. The results of doripenem monotherapy (A), polymyxin B monotherapy (B), doripenem at 500 mg every 8 h in combination with various clinical doses of polymyxin B (C), doripenem at 500 mg every 8 h in combination with various polymyxin B "burst" doses (PB dosing only for 1 day) (D), doripenem at 750 mg every 8 h in combination with various polymyxin B burst doses (PB dosing only for 1 day) (E), and doripenem at 1,000 mg every 8 h in combination with various polymyxin B burst doses (PB dosing only for 1 day) (F) are shown.

error (i.e., 10^{-9} to 10^{-8} mutations per generation) and lack of DNA repair mechanisms (9). Li et al. determined that deactivation of DNA mismatch repair also helps to prolong bacterial survival (7). Although mutations in DNA repair mechanisms that lead to the mutator phenotype have been well characterized in species such as *Escherichia coli* and *P. aeruginosa* (10, 34), there is a scarcity of data regarding antibiotic pharmacodynamics against these pathogens.

Polymyxin B is an "old" antibiotic (11–14) that was reintroduced into the clinic for treating Gram-negative infections resistant to essentially all other antibiotics, although the emergence of polymyxin resistance when used as monotherapy is alarming (15–22). Despite an anticipated pathogenicity cost to attain polymyxin B resistance in some subsets of the infecting *P. aeruginosa* population (i.e., *mutS*), these chronic infections still can be deadly (8). Rationally optimized combination therapy with polymyxins offers a highly promising approach to overcome resistance, especially against the mutator phenotype (16, 19, 23–27, 52). Here, we have utilized *in vitro* time-kill experiments and mechanism-based mathematical models to evaluate and optimize polymyxin B based combination therapies against *P. aeruginosa* mutators.

Monotherapy regimens at the highest polymyxin B and doripenem concentrations (exceeding the clinically achievable unbound concentrations) were unable to eradicate the wild type and its isogenic mutator strains at 10^8 CFU/ml. However, our results at a lower inoculum of 10^6 CFU/ml showed that polymyxin B monotherapy achieved bactericidal activity at clinically relevant concentrations despite attenuated killing against mutator strains. These data are similar to other studies that show a marked inoculum effect for *P. aeruginosa* (16, 20), and therefore a combination with doripenem was assessed against a 10^8 CFU/ml starting inoculum. The higher bacterial density of 10^8 CFU/ml was chosen to simulate severe bacterial pneumonia (53, 54). Our findings for polymyxin B and doripenem combination are in agreement with the observations of our group and others against resistant Gram-negative pathogens studied *in vitro* and *in vivo* (15, 16, 18–20). Additionally, recent clinical studies suggest that the currently approved dosing regimens for polymyxin B monotherapy may be sufficient

to treat infections caused by pathogens that exhibit a polymyxin B MIC of ≤2 mg/liter (likely unsuitable for higher MICs). The dosing in these studies was based on simulations that assume a target *f*AUC/MIC ratio of 20 (55, 56). However, based on our simulations, monotherapy with polymyxin B against the wild-type strain with an MIC of 1 mg/liter is likely not suitable for treatment of serious infections with a high bacterial inoculum. The discrepancy in the predicted efficacy of monotherapy may be attributed to this difference in the initial inoculum (10^8 CFU/ml for our *in vitro* study versus $10^{6.2}$ to $10^{6.8}$ CFU/g [55]) and the differences in the experimental model (*in vitro* versus *in vivo*) used.

The mutator strains displayed a high fraction of resistant subpopulations prior to treatment, and therefore the rapid rate of regrowth during antibiotic exposure can likely be attributed to the higher number of preexisting resistant bacteria. This phenomenon has been confirmed by our mathematical models and can also be explained by multiple molecular mechanisms. A deficient DNA repair system coupled with natural mutations resulting from replication errors (9) contribute to a high probability for generation of both favorable and undesirable mutations each replication cycle. The presence of an antibiotic acting as a selective agent leads to evolutionary change toward the resistant phenotype. Additionally, regrowth could also be attributed to adaptive resistance whereby bacteria adapt to their environment by modulating gene expression. Two-component regulators, phoP-phoQ (57), pmrA-pmrB (58), and ParR-ParS (59) have been associated with the adaptive resistance in Gram-negative infections upon exposure to polymyxins. Collectively, the inability of polymyxin B monotherapy to eradicate *P. aeruginosa* PAO1 and mutator strains may be due to multiple mechanisms; however, provided that each experiment was relatively short in duration and was initiated at a high inoculum, amplification of one or more preexisting populations is the most probable cause of regrowth.

Similar to other studies, our findings showed that polymyxin B activity was attenuated at a high initial inoculum where rapid regrowth occurred even with the highest dose of polymyxin B (64 mg/liter) against all strains (15, 16, 18–20, 39). Although doripenem monotherapy displayed substantial killing of all strains, it was unable to eradicate bacteria even at the highest doripenem concentration (up to 50 mg/liter; see Fig. S2 in the supplemental material). This observation could result from the downregulation of the OprD porin (a channel for doripenem to permeate to the site of action) (60).

A previously developed PD model for the combination of colistin and doripenem was successfully applied as a platform to develop current PD models for the mutator strains (19). Both "subpopulation" and "mechanistic" synergy were considered based on our previous model. Our experimental data and mechanism-based models indicated greatly enhanced killing between polymyxin B and doripenem. We thus explored the potential benefit of this combination at clinically relevant concentrations and novel dosage regimens for both antibiotics.

Collectively, these results suggest the benefit of using polymyxin-based combinations against all strains of *P. aeruginosa*, which is in agreement with previous studies of colistin against nonmutator *P. aeruginosa* strains (16, 19). The wild-type strain required lower concentrations of polymyxin B (4 mg/liter versus 8 mg/liter) in combination with doripenem to achieve suppression of growth compared to all mutator strains. However, high free concentrations of polymyxin B (i.e., ≥4 mg/liter) may not be achievable based on current clinical dosing. Clinically, polymyxin B is dosed at 2.5 to 3 mg/kg daily for up to 14 days. The dose of 1.5 mg/kg every 12 h achieves a maximum concentration (C_{max}) of 8.51 mg/liter or free C_{max} of 3.57 mg/liter (free fraction of 0.42) and an average steady-state concentration ($C_{ss,avg}$) of 2.79 mg/liter (49). Such concentrations may not be achievable to maximize the enhanced killing with high doripenem concentrations against the wild-type and especially mutator strains. Alternative dosing strategies and different drug combinations are needed to combat such infections.

PK/PD simulations were conducted to investigate the utility of alternative dosing strategies. Our simulations showed that monotherapy when given in a traditional fashion completely regrew against a high bacterial density of *P. aeruginosa* using the population PK for polymyxin B or doripenem. In contrast, the traditional clinical polymyxin B regimens and novel front-loaded dosing regimens in combination with doripenem were predicted to achieve good killing and minimize resistance against the wild-type strain. However, the combinations were less effective against the *mutS* strain. To explore other novel regimens, we simulated a "burst" regimen of high intensity polymyxin B (2.5, 5, 7.5, and 10 mg/kg) administered over only the first 24 h in combination with doripenem. The result was significantly improved bacterial killing compared to the combination therapy with standard polymyxin B dosing. With the "burst" dosing strategy of polymyxin B in combination with doripenem, the total dose of polymyxin B for a 7-day treatment course is 1.75- to 3.5-fold lower than the standard regimens (i.e., the total polymyxin B "burst" dose is 2.5 to 10 mg/kg and dose based on the standard dosage regimen of 1.25 mg/kg every 12 h is 17.5 mg/kg).

The major concern for polymyxin usage is dose-limiting nephrotoxicity (61, 62). Colistin-associated nephrotoxicity is well correlated with the colistin $C_{ss,avg}$ or C_{min} and has a median onset of nephrotoxicity of about 2 days (63–65). Although polymyxin B nephrotoxicity may be less than that of colistin, there is still a significant proportion of drug-induced nephrotoxicity (66, 67). New dosing strategies in which a high intensity of polymyxin B is administered during the first day, followed by no additional polymyxin doses, may offer the advantage of reducing toxicity and maximizing bacterial killing. Furthermore, polymyxin B is administered as an active drug (unlike colistin), making high polymyxin B plasma concentrations more rapidly achievable. Further proof of concept studies in animal models (such as a rabbit model) are needed to assess the utility and nephrotoxicity of combinations via "front-loading" and "burst" regimens. This lack of validation of our PK/PD simulations in an *in vivo* system are a limitation of this study. The PK/PD indices of our work may not directly translate to an immunocompetent host who has a robust immune response to aid in the elimination of infection. For example, although bacterial eradication in the *in vitro* system may predict clinical success in both immunocompetent and immunocompromised hosts, microbiologic cure in a patient with an operational immune system could occur with bactericidal killing and no eradication.

In summary, experiments discussed here demonstrate that the mutator phenotype in *P. aeruginosa* is particularly difficult to treat and requires high and potentially dangerous drug exposure if antibiotics are used as monotherapy. We demonstrated enhanced killing by polymyxin B-based combinations with doripenem against a high bacterial density infection *in vitro* against mutator strains. Modeling and simulation provided a quantitative ap-

proach for optimizing polymyxin B-based therapy against mutator strains and generated predictions for the bacterial killing by novel and clinically relevant combination dosage regimens. However, the relative dose intensity, timing, and optimization of each agent in combination is the subject of future validation studies. Further investigation using *in vitro* hollow-fiber and *in vivo* animal infection models with the proposed combination regimens are necessary prior to translating these dosing strategies to patients.

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