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The Combination of Colistin and Doripenem Is Synergistic against *Klebsiella pneumoniae* at Multiple Inocula and Suppresses Colistin Resistance in an *In Vitro* Pharmacokinetic/Pharmacodynamic Model

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Multidrug-resistant (MDR) *Klebsiella pneumoniae* may require combination therapy. We systematically investigated bacterial killing with colistin and doripenem mono- and combination therapy against MDR *K. pneumoniae* and emergence of colistin resistance. A one-compartment *in vitro* pharmacokinetic/pharmacodynamic model was employed over a 72-h period with two inocula ($\sim 10^6$ and $\sim 10^8$ CFU/ml); a colistin-heteroresistant reference strain (ATCC 13883) and three clinical isolates (colistin-susceptible FADDI-KP032 [doripenem resistant], colistin-heteroresistant FADDI-KP033, and colistin-resistant FADDI-KP035) were included. Four combinations utilizing clinically achievable concentrations were investigated. Microbiological responses were examined by determining log changes and population analysis profiles (for emergence of colistin resistance) over 72 h. Against colistin-susceptible and -heteroresistant isolates, combinations of colistin (constant concentration regimens of 0.5 or 2 mg/liter) plus doripenem (steady-state peak concentration [C_{max}] of 2.5 or 25 mg/liter over 8 h; half-life, 1.5 h) generally resulted in substantial improvements in bacterial killing at both inocula. Combinations were additive or synergistic against ATCC 13883, FADDI-KP032, and FADDI-KP033 in 9, 9, and 14 of 16 cases (4 combinations at 6, 24, 48, and 72 h) at the 10^6-CFU/ml inoculum and 14, 11, and 12 of 16 cases at the 10^8-CFU/ml inoculum, respectively. Combinations at the highest dosage regimens resulted in undetectable bacterial counts at 72 h in 5 of 8 cases (4 isolates at 2 inocula). Emergence of colistin-resistant subpopulations in colistin-susceptible and -heteroresistant isolates was virtually eliminated with combination therapy. Against the colistin-resistant isolate, colistin at 2 mg/liter plus doripenem (C_{max}, 25 mg/liter) at the low inoculum improved bacterial killing. This investigation provides important information for optimization of colistin-doripenem combinations.

The emergence of nosocomial *Klebsiella pneumoniae* resistant to almost all available antibiotics, including carbapenems, is an increasing problem worldwide (7, 18, 21, 22, 27, 38). Increasing resistance combined with a lack of novel antibiotics in the drug discovery pipeline for Gram-negative pathogens (7) has forced a reevaluation of "old" antibiotics, in particular colistin (polymyxin E), which retains activity against many of these multidrug-resistant (MDR) Gram-negative organisms (24, 30, 31, 33). As a consequence, the use of colistin has increased substantially over the past few years, particularly for critically ill patients. However, colistin-resistant *K. pneumoniae* has rapidly emerged (1, 6, 26, 35, 48).

Colistin is administered parenterally in the form of its sulfomethyl derivative, sodium colistin methanesulfonate (CMS). The inactive prodrug CMS undergoes conversion *in vivo* to the active species, colistin (4, 17, 43). Although CMS has been available in the clinic for more than 50 years, only recently has reliable information on the pharmacokinetics (PK) and pharmacodynamics (PD) of CMS and formed colistin emerged. It is evident from these investigations that the plasma colistin concentrations achieved in critically ill patients with currently recommended CMS dosage regimens are low and in many cases suboptimal (17, 43). Unfortunately, colistin-induced nephrotoxicity is a dose-limiting adverse effect in up to ~50% of patients (11, 17, 19, 28), and thus increasing the daily dose may not be an acceptable option (17). Further complicating the use of CMS as monotherapy is the phenomenon of colistin heteroresistance, the presence of colistin-resistant subpopulations within an isolate that is susceptible based upon its MIC. Colistin heteroresistance, which has been identified in *K. pneumoniae* (37, 44), *Acinetobacter baumannii* (20, 32, 51), and *Pseudomonas aeruginosa* (3), is thought to contribute to the rapid emergence of colistin resistance observed with monotherapy. Collectively, these observations suggest caution with the use of colistin monotherapy and have led some to propose that colistin may be best used as part of combination therapy (17, 40). The aim of the present study was to systematically investigate using a range of isolates the extent of *in vitro* bacterial killing and the emergence of colistin resistance of *K. pneumoniae*, at both low and high inocula, using clinically relevant dosage regimens of colistin alone and in combination with doripenem.

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TABLE 1 MICs for *K. pneumoniae* isolates used in this study[a]

Isolate	MIC (mg/liter)[b]		β-Lactamase typing[c]	Colistin heteroresistant[d]
	Colistin	Doripenem		
ATCC 13883	1	<0.125	SHV	Yes
FADDI-KP033[e]	1	<0.125	SHV and DHA	Yes
FADDI-KP032[e]	1	8	SHV and DHA	No
FADDI-KP035	>128	<0.125	SHV	NA

[a] All clinical isolates were MDR according to the definition of the Centers for Disease Control and Prevention (45).
[b] EUCAST breakpoints for colistin are ≤2 mg/liter for susceptibility and >2 mg/liter for resistance. For doripenem, the breakpoints are ≤1 mg/liter for susceptibility and >4 mg/liter for resistance (14).
[c] The strain contains genes encoding SHV class A and/or DHA AmpC type β-lactamase.
[d] Heteroresistance to colistin was defined as the existence, in an isolate for which the colistin MIC was ≤2 mg/liter, of subpopulations able to grow in the presence of >2 mg/liter colistin (44). NA, not applicable due to colistin resistance.
[e] FADDI-KP033 and FADDI-KP032 were taken from the same patient before and after carbapenem therapy (isolates 16 and 17 from a previous study [44]).

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MATERIALS AND METHODS

Bacterial isolates. *K. pneumoniae* ATCC 13883 (American Type Culture Collection, Rockville, MD) and three clinical isolates (FADDI-KP032, FADDI-KP033, and FADDI-KP035) were selected based upon differing patterns of susceptibility to colistin and doripenem and included colistin-heteroresistant strains (Table 1); the method of determining colistin heteroresistance is described below. FADDI-KP033 and FADDI-KP032 were kindly donated by Ben Howden (Austin Hospital, Melbourne, Australia) and were taken from the same patient before and after carbapenem therapy, whereas FADDI-KP035 was from Diagnostic Services of Manitoba (Health Sciences Centre, Winnipeg, Canada). All clinical strains in this study fulfilled the definition of MDR of the Centers for Disease Control and Prevention (45).

MICs of colistin and doripenem were determined for each isolate in two replicates on separate days in cation-adjusted Mueller-Hinton broth (CAMHB) (containing 23.0 mg/liter Ca^{2+} and 11.5 mg/liter Mg^{2+} [Oxoid, Hampshire, England]) via broth microdilution (15); European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Växjö, Sweden) breakpoints against the *Enterobacteriaceae* were employed (Table 1) (14). All isolates produced β-lactamases, although only FADDI-KP032 was resistant to doripenem based upon MICs (Table 1). Isolates were stored in tryptone soy broth (Oxoid, Basingstoke, Hampshire, England) with 20% glycerol (Ajax Finechem, Seven Hills, NSW, Australia) at −80°C.

Antibiotics. For MIC determinations and *in vitro* PK/PD studies, colistin sulfate (lot 070M1499V; 23,690 U/mg) was purchased from Sigma-Aldrich (St. Louis, MO), while doripenem (lot 9GBSE00) was from Janssen-Cilag (Australia). Colistin sulfate was employed in the current study because colistin is the active antibacterial agent formed *in vivo* following administration of CMS (4). Colistin was prepared using Milli-Q water (Millipore Australia, North Ryde, New South Wales, Australia) at the beginning of each experiment and spiked into the sterile broth that was pumped into the central reservoir (see below) to achieve the desired concentration; we have previously shown that colistin is stable under these conditions for the duration of the experiment (5). Stock solutions of doripenem were similarly prepared using 0.9% sodium chloride immediately prior to each administration to the PK/PD model, protected from light to minimize loss from degradation, and sterilized by filtration with a 0.22-μm Millex-GP filter (Millipore, Bedford, MA). Concentrations of colistin and doripenem in the reservoirs were measured using high-performance liquid chromatography as described previously (5, 29).

***In vitro* pharmacokinetic/pharmacodynamic model.** A one-compartment *in vitro* PK/PD model was used to examine the microbiological response and emergence of resistance to various dosage regimens of colistin and doripenem alone and in combination over 72 h at two different starting inocula (~10^6 and ~10^8 CFU/ml) (5). All drug-containing regimens (whether mono- or combination therapy) mimicked the PK profiles of each drug achieved in critically ill patients (17, 39, 43). Since we have previously demonstrated that both colistin (2) and doripenem (5) are almost entirely unbound in CAMHB, the specified concentrations represent unbound (free) concentrations. Colistin (when included in the dosage regimen) was delivered at a clinically relevant concentration (Table 2) to mimic the flat colistin concentration-versus-time profiles observed across a dosage interval at steady state in patients receiving short-term infusions of CMS intravenously (i.v.) every 8 to 24 h (17, 43); since colistin was added to all media prior to each experiment, steady-state concentrations were present from the beginning of each experiment. For doripenem-containing regimens, doripenem was injected as a bolus dose into each treatment compartment following bacterial inoculation to achieve the desired steady-state peak concentration (C_{max}), with intermittent dosing every 8 h thereafter (Table 2); no loading dose of doripenem was required to achieve steady-state concentrations, since doripenem does not accumulate with intermittent i.v. administration. The flow rate of sterile media through the system simulated a doripenem elimination half-life ($t_{1/2}$) of 1.5 h, which approximates that in critically ill patients (36). Three monotherapy dosage regimens (each) were simulated for colistin and doripenem (for the colistin-resistant isolate, only colistin at 5 mg/liter was used), with four colistin-doripenem regimens used for combination therapy (Table 2).

Microbiological response and emergence of resistance to colistin. Serial samples (1 ml) were collected aseptically from the central reservoir of the PK/PD model at the times shown in Table 2 for determination of colistin and doripenem concentrations, as well as viable cell counting and real-time population analysis profiles (PAPs) (5). Viable counting and PAPs were conducted immediately after sampling by spiral plating (WASP2 spiral plater; Don Whitley Scientific Ltd., United Kingdom) 50 μl of sample (appropriately diluted with 0.9% saline) on either nutrient agar (viable counting) or Mueller-Hinton agar (PAPs), followed by incubation for 24 h (48 h for plates with small colonies) at 35°C. The limit of detection was 20 CFU/ml (equivalent to 1 colony per plate), whereas the limit of quantification was 400 CFU/ml (equivalent to 20 colonies per plate). Colistin heteroresistance was defined as a colistin-susceptible isolate (i.e., MIC ≤ 2 mg/liter) in which subpopulations were able to grow in the presence of >2 mg/liter colistin in the PAPs (44).

PD analysis. The log change method comparing the change in $\log_{10}$ (CFU/ml) from 0 h (CFU_0) to time t (6, 24, 48, or 72 h; CFU_t), calculated as log change = $\log_{10} CFU_t - \log_{10} CFU_0$, was used to examine microbiological responses to monotherapy and combination therapy. Single-antibiotic or combination regimens causing a reduction of ≥1 $\log_{10}$ CFU/ml below the initial inoculum at the specified time were considered active. Synergy was defined as ≥2-$\log_{10}$ killing for the combination relative to that for its most active component at the specified time; additivity was defined as a 1- to <2-$\log_{10}$ superior killing for the combination (42).

RESULTS

PK validation. The colistin concentrations achieved (mean ± standard deviation [SD]) were 0.48 ± 0.13 mg/liter (n = 40), 1.92 ± 0.35 mg/liter (n = 46), and 5.11 ± 0.83 mg/liter (n = 19) for the targeted concentrations of 0.50, 2.00, and 5.00 mg/liter, respectively. The measured doripenem C_{max} was 2.84 ± 0.41 mg/liter (n = 52) for the targeted value of 2.50 mg/liter, 24.5 ± 2.78 mg/liter (n = 56) for the targeted value of 25.0 mg/liter, and 49.0 ± 4.06 mg/liter (n = 17) mg/liter for the targeted value of 50.0 mg/liter.

TABLE 2 Colistin and doripenem dosage regimens, PK/PD index values, and sampling times in the *in vitro* PK/PD model[a]

Treatment regimen	PK/PD index value[b]												Sampling times (h) for microbiological measurements[f]
	*f*AUC/MIC				*f*C_{max}/MIC				*f*$T_{>MIC}$				
	ATCC 13883	FADDI-KP033	FADDI-KP032	FADDI-KP035	ATCC 13883	FADDI-KP033	FADDI-KP032	FADDI-KP035	ATCC 13883	FADDI-KP033	FADDI-KP032	FADDI-KP035	
Col monotherapy,[c] target concn (mg/liter)													0, 1, 2, 3, 4, 6, 8, 24, 26, 28, 48, 50, 52, 72
0.5	12.0	12.0	12.0	<0.09	0.50	0.50	0.50	<0.004	0	0	0	0	
2.0	48.0	48.0	48.0	<0.38	2.0	2.0	2.0	<0.016	100	100	100	0	
5.0	120.0	120.0	120.0	<0.94	5.0	5.0	5.0	<0.039	100	100	100	0	
Dor monotherapy,[d] target C_{max}/C_{min} (mg/liter)													0, 1, 2, 3, 4, 6, 8, 24, 26, 28, 48, 50, 52, 72
2.5/0.062	>127	>127	1.98	>127	>20	>20	0.32	>20	81.0	81.0	0	81.0	
25/0.62	>1,266	>1,266	19.8	>1,266	>200	>200	3.13	>200	100	100	30.8	100	
50/1.24	>2,532	>2,532	39.6	>2,532	>400	>400	6.25	>400	100	100	49.6	100	
Combination therapy[e]													0, 1, 2, 3, 4, 6, 8, 24, 26, 28, 48, 50, 52, 72

[a] Dosage regimens were tested with ~10^6- and ~10^8-CFU/ml starting inocula.
[b] Values shown are target values for PK/PD indices. For combination therapy, the PK/PD indices for each drug were the same as those for equivalent monotherapy. *f*AUC/MIC, area under the unbound drug concentration-time curve over 24 h in the steady state divided by the MIC; *f*C_{max}/MIC, unbound drug maximal concentration divided by the MIC; *f*$T_{>MIC}$, cumulative percentage of a 24-h period that the unbound drug concentration exceeds the MIC under steady-state PK conditions.
[c] Colistin (Col) dosage regimens involved a constant concentration of colistin simulating the flat colistin concentration-versus-time profiles observed across a dosing interval at steady state in patients receiving short-term infusions of CMS i.v. every 8 to 24 h. For the colistin-resistant isolate (FADDI-KP035), only colistin at 5 mg/liter was used as monotherapy. Values shown for isolate FADDI-KP035 at other dosages of colistin are those for combination therapy with the indicated dosage regimen of colistin.
[d] Doripenem (Dor) dosage regimens involved intermittent administration (every 8 h) to achieve the targeted C_{max} and C_{min}.
[e] Combination therapy was carried out with the following dosage regimens: colistin at 0.5 mg/liter plus a doripenem C_{max} of 2.5 mg/liter, colistin at 0.5 mg/liter plus a doripenem C_{max} of 25 mg/liter, colistin at 2 mg/liter plus a doripenem C_{max} of 2.5 mg/liter, and colistin at 2 mg/liter plus a doripenem C_{max} of 25 mg/liter.
[f] The number of CFU/ml was determined at all time points. Full PAPs were generated at 0 and 72 h; mini-PAPs were generated at 6, 24, and 48 h.

Microbiological response. For the targeted inoculum of ~10^6 CFU/ml, initial $\log_{10}$ CFU/ml (mean ± SD) were 6.21 ± 0.19 (n = 11), 6.54 ± 0.25 (n = 11), 6.48 ± 0.19 (n = 11), and 6.56 ± 0.18 (n = 9) for ATCC 13883, FADDI-KP033, FADDI-KP032, and FADDI-KP035, respectively; the corresponding values for the targeted inoculum of ~10^8 CFU/ml were 8.17 ± 0.15 (n = 11), 7.94 ± 0.08 (n = 11), 8.28 ± 0.13 (n = 11), and 8.31 ± 0.16 (n = 8) $\log_{10}$ CFU/ml. The colistin PAPs conducted at baseline at an inoculum of ~10^8 CFU/ml revealed the presence of preexisting colistin-resistant subpopulations (i.e., colistin heteroresistance) in some isolates (Fig. 1, baseline). The time course profiles of bacterial numbers achieved with all dosage regimens for each of the four isolates at both inocula are shown in Fig. 1 and 2. Log changes in viable cell counts at each inoculum with mono- and combination therapy are presented in Table 3.

Colistin monotherapy. At the 10^6-CFU/ml inoculum, colistin monotherapy regimens (a constant concentration of 0.5, 2, or 5 mg/liter) against the colistin-susceptible and -heteroresistant isolates produced rapid and extensive initial killing to ~2-$\log_{10}$ CFU/ml or below (Fig. 1). For the colistin-susceptible isolate KP032 and the heteroresistant clinical isolate KP033, no viable bacteria were detected by 1 to 2 h with colistin at 2 and 5 mg/liter (and colistin at 0.5 mg/liter for KP033). For KP032, regrowth equivalent to that of the control occurred by 48 h with colistin at 0.5 mg/liter, whereas regrowth with the higher colistin concentration regimens was substantially reduced across the 72 h. For both heteroresistant strains, regrowth approaching that of the control occurred by 24 to 48 h with all colistin regimens (0.5, 2, and 5 mg/liter). An inoculum effect with colistin monotherapy was evident. At the 10^8-CFU/ml inoculum, colistin at 0.5 and 2 mg/liter produced virtually no bacterial killing at any time against the colistin-susceptible or -heteroresistant strains. Although colistin at 5 mg/liter produced rapid and extensive initial killing (~5- to 6-$\log_{10}$ CFU/ml) against these isolates, regrowth was evident by 4 to 6 h, and bacterial counts had returned close to control values by 24 h in all cases (Fig. 2). Against the colistin-resistant isolate KP035, bacterial growth in the presence of colistin at 5 mg/liter was essentially no different from that of the growth control at either inoculum (Fig. 2).

Doripenem monotherapy. For doripenem-susceptible isolates at the 10^6-CFU/ml inoculum, all doripenem regimens (C_{max} of 2.5, 25, or 50 mg/liter) produced rapid initial bacterial killing by ≥~3-$\log_{10}$ CFU/ml. For ATCC 13883, with a doripenem C_{max} of 2.5 mg/liter, regrowth had begun by 6 h, and bacterial numbers remained ~3-$\log_{10}$ CFU/ml below control values across the 72 h (Fig. 1); regrowth with doripenem C_{max} values of 25 and 50 mg/liter was greatly suppressed for this strain, with no viable bacteria detected from 48 h onwards. For the remaining two doripenem-susceptible isolates, regrowth with doripenem C_{max} values of 2.5 and 25 mg/liter was evident by 8 h and was within ~1- to 3-$\log_{10}$ CFU/ml of control values by 72 h. With the highest doripenem dosage regimen (C_{max} of 50 mg/liter), no viable bacteria were detected by 2 to 6 h, although substantial regrowth beginning at 24 h occurred for KP035; for KP033, no viable bacteria were detected after 2 h. For the doripenem-resistant isolate (KP032), doripenem C_{max} values of 25 and 50 mg/liter each produced substantial early bacterial killing despite a doripenem MIC of 8 mg/liter, with regrowth to ~2-$\log_{10}$ CFU/ml by 72 h; bacterial growth in the pres-

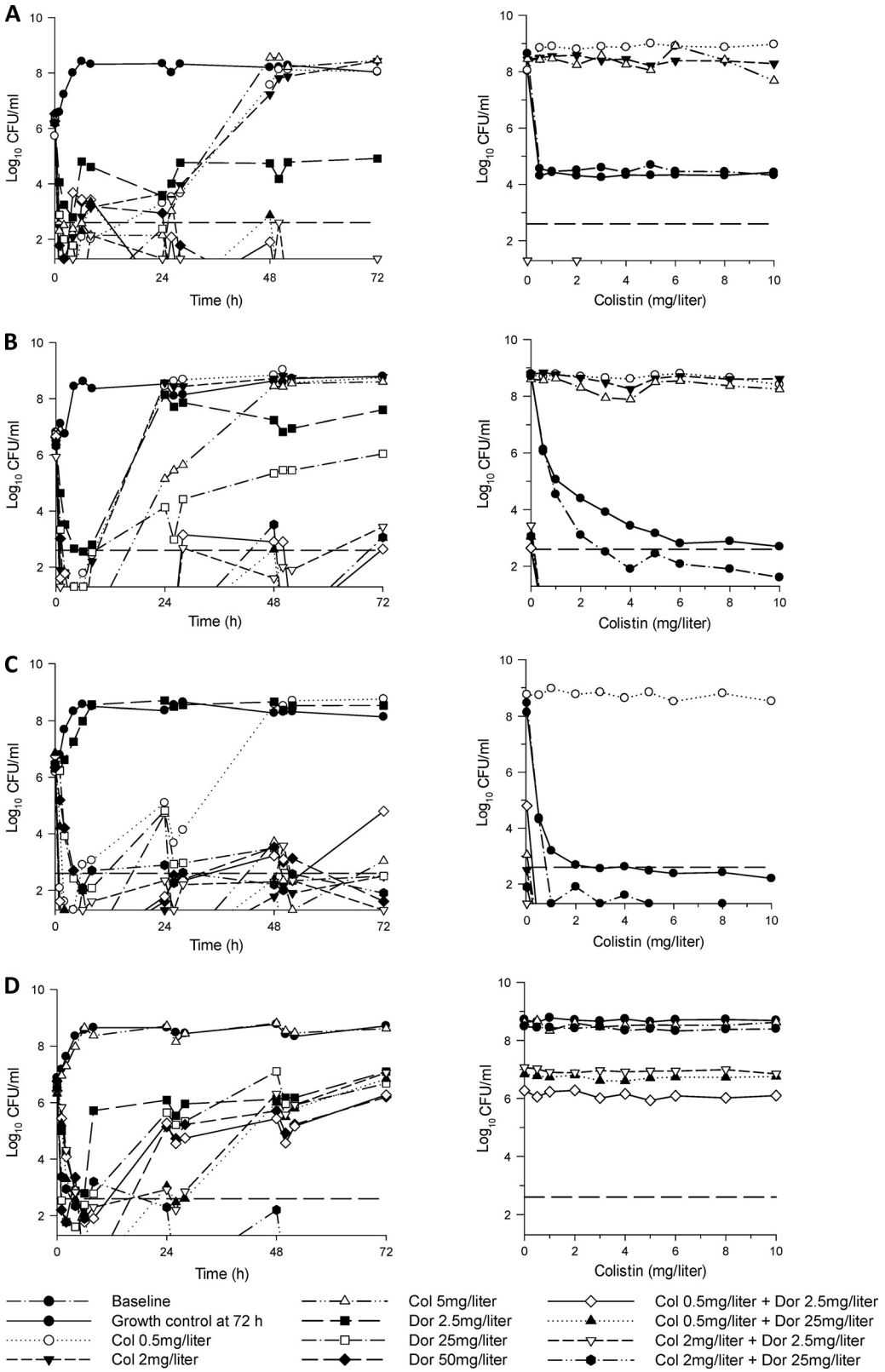


TABLE 3 Log changes in numbers of CFU[a]

Isolate	Inoculum (CFU/ml)	Time (h)	Log change (= $\log_{10}(CFU_t) - \log_{10}(CFU_0)$)									
			Col 0.5mg/liter	Col 2mg/liter	Col 5mg/liter	Dor 2.5mg/liter	Dor 25mg/liter	Dor 50mg/liter	Col 0.5mg/liter + Dor 2.5mg/liter	Col 0.5mg/liter + Dor 25mg/liter	Col 2mg/liter + Dor 2.5mg/liter	Col 2mg/liter + Dor 25mg/liter
ATCC 13883	10^6	6	-3.64	-3.37	-3.61	-1.47	-6.39	-3.16	-2.83	-3.89	-6.19	-6.19
		24	-2.42	-2.55	-4.04	-2.72	-4.01	-3.58	-6.26	-6.19	-4.89	-6.19
		48	1.84	1.04	2.36	-1.54	-6.39	-6.53	-4.36	-3.32	-6.19	-6.19
		72	2.33	2.25	2.26	-1.36	-6.39	-6.53	-6.26	-6.19	-4.89	-6.19
	10^8	6	0.23	-0.34	-2.68	-2.71	-4.10	-5.11	-2.21	-5.34	-0.96	-5.21
		24	0.28	-0.47	-0.09	-2.16	-3.20	-3.16	-3.74	-5.00	-5.01	-7.14
		48	0.24	-0.76	0.17	-1.99	-2.78	-2.63	-6.41	-8.20	-8.07	-8.44
		72	0.07	-1.09	0.13	-2.84	-4.70	-2.96	-6.19	-8.20	-8.07	-8.44
FADDI- KP033	10^6	6	-4.82	-6.43	-6.81	-4.03	-5.43	-6.63	-6.66	-6.44	-5.93	-6.33
		24	1.83	2.14	-1.67	1.56	-2.60	-6.63	-6.66	-6.44	-5.93	-6.33
		48	2.23	2.28	1.64	0.65	-1.39	-6.63	-3.76	-3.82	-4.33	-2.81
		72	2.14	2.34	1.79	1.02	-0.69	-6.63	-4.02	-3.40	-2.50	-3.26
	10^8	6	1.18	1.11	-4.35	-2.16	-5.00	-3.25	-1.71	-6.63	-1.41	-6.39
		24	0.89	1.12	1.13	-1.69	-2.20	-1.65	-2.71	-4.80	-2.63	-8.00
		48	0.80	0.91	0.67	-0.28	-0.87	-1.06	-2.89	-7.94	-3.54	-8.00
		72	0.91	1.10	0.92	-0.56	-0.96	-0.89	-1.04	-6.63	-2.72	-8.00
FADDI- KP032	10^6	6	-3.28	-6.48	-6.48	1.67	-4.43	-4.34	-6.75	-6.86	-5.18	-4.40
		24	-1.09	-5.18	-1.77	2.39	-1.61	-4.74	-4.97	-6.86	-4.13	-3.59
		48	2.39	-4.70	-2.76	2.35	-2.94	-2.82	-3.53	-4.48	-4.18	-4.27
		72	2.57	-3.97	-3.44	2.22	-3.92	-4.74	-1.95	-6.86	-5.18	-4.57
	10^8	6	0.38	0.06	-4.73	-0.40	-3.98	-5.61	0.36	-4.78	-3.97	-5.64
		24	0.38	0.12	0.24	0.17	-1.35	-5.41	0.25	-6.63	-1.76	-5.99
		48	0.38	-0.31	0.06	0.21	-1.45	-4.35	0.23	-6.93	-2.78	-6.59
		72	0.23	-0.12	0.23	0.30	-1.57	-5.13	0.30	-6.33	-2.06	-8.19
FADDI- KP035[b]	10^6	6	-	-	2.14	-3.77	-4.29	-5.21	-4.64	-4.24	-4.54	-4.61
		24	-	-	2.21	-0.48	-1.02	-6.52	-1.15	-3.28	-3.52	-4.21
		48	-	-	2.31	-0.44	0.44	-6.52	-0.99	-0.32	-0.14	-4.31
		72	-	-	2.12	0.53	0.01	-0.16	-0.15	0.52	0.62	-6.52
	10^8	6	-	-	0.28	-2.06	-2.87	-3.00	NA	-3.33	-2.31	-3.53
		24	-	-	0.09	-1.42	-2.31	-2.80	NA	-2.15	-1.85	-2.56
		48	-	-	0.03	-1.21	-1.51	-1.99	NA	-1.93	-1.83	-1.42
		72	-	-	0.04	-1.35	-1.32	-2.37	NA	-0.91	-1.18	-1.42

[a] Log changes at 6, 24, 48, and 72 h at an inoculum of 10^6 or 10^8 CFU/ml with colistin (Col) and/or doripenem (Dor) against four *K. pneumoniae* isolates. The gray background indicates activity (a reduction of $\geq$1-$\log_{10}$ CFU/ml below the initial inoculum); the green background indicates synergy (a $\geq$2-$\log_{10}$ decrease in the number of CFU/ml between the combination and its most active component); the red background indicates additivity (a 1.0- to <2-$\log_{10}$ decrease in the number of CFU/ml between the combination and its most active component). NA, not available.

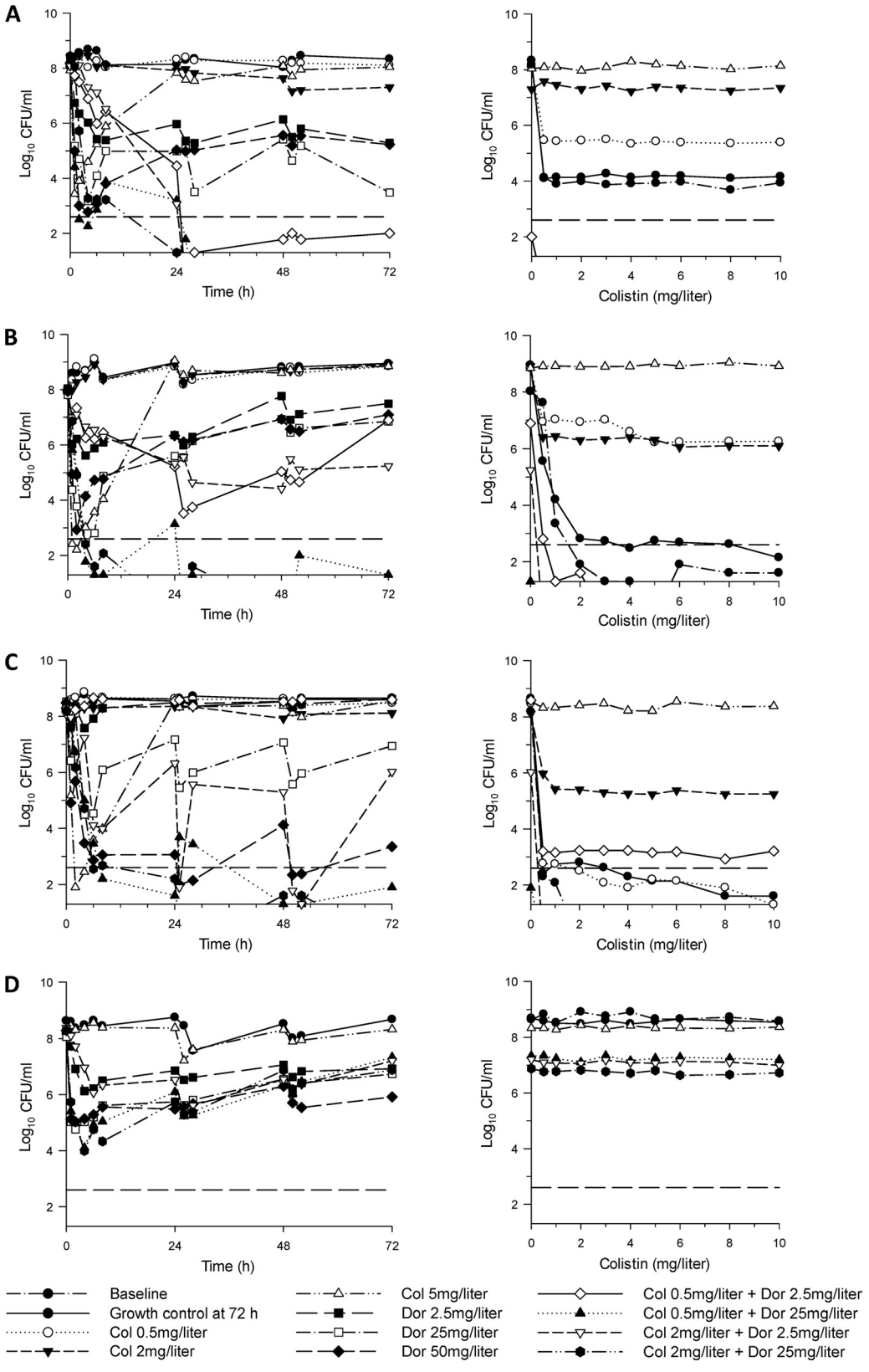
[b] Colistin-resistant isolate; colistin monotherapy performed with 5 mg/liter only. The calculations of synergy/additivity were based on colistin at 5 mg/liter.

ence of a doripenem C_{max} of 2.5 mg/liter was essentially no different from that of the growth control. An inoculum effect was generally observed with monotherapy against doripenem-susceptible isolates.

Combination therapy. For the colistin-susceptible isolate KP032, the addition of doripenem at a C_{max} of 2.5 or 25 mg/liter to colistin at 0.5 mg/liter at the low inoculum produced ~1.5-$\log_{10}$-greater initial killing than monotherapy, with no viable bacteria detected from 2 to 4 h; both combinations resulted in synergy at virtually all time points across 72 h. Improved activity was particularly noticeable with the combination of colistin at 0.5 mg/liter and doripenem at 2.5 mg/liter, which produced ~4- to 5-$\log_{10}$-greater killing than monotherapy at 48 and 72 h. There was no enhancement of bacterial activity at this inoculum with combinations containing colistin at 2 mg/liter, with similarly large activity compared to that in the presence of equivalent colistin monotherapy (Fig. 1C and Table 3). At the 10^8-CFU/ml inoculum, bacterial killing was not improved when colistin and doripenem were combined in the lowest-dosage regimens (constant 0.5 mg/liter of colistin and a doripenem C_{max} of 2.5 mg/liter); however, all other combinations resulted in substantially greater activity with synergistic (predominantly) or additive effects at nearly all time points (Fig. 2C and Table 3). Combinations with colistin at 0.5 or 2 mg/liter plus a doripenem C_{max} of 25 mg/liter were particularly active and produced ~5- to 7-$\log_{10}$-greater killing than the most active monotherapy (doripenem) at 48 and 72 h, with no viable colonies detected across the 72-h period on at least one occasion.

Against both colistin-heteroresistant strains, bacterial killing was substantially enhanced at most time points across 72 h with most combinations at both inocula (Fig. 1 and 2 and Table 3). For

FIG 1 (Left) Time-kill curves with various clinically relevant dosage regimens of colistin (Col) and doripenem (Dor) alone and in combination at an inoculum of ~10^6 CFU/ml. (Right) PAPs at baseline and after 72 h of exposure to colistin monotherapy, colistin-doripenem combination therapy, or neither antibiotic (growth control). Doripenem monotherapy regimens were not included in colistin-PAP examination. (A) ATCC 13883 (colistin heteroresistant, doripenem susceptible, MDR); (B) FADDI-KP033 (colistin heteroresistant, doripenem susceptible, MDR); (C) FADDI-KP032 (colistin susceptible, doripenem resistant, MDR); (D) FADDI-KP035 (colistin resistant, doripenem susceptible, MDR). The *y* axis starts at the limit of detection, and the limit of quantification is indicated by the dashed horizontal line.



clinical isolate KP033 at the low inoculum, combinations containing a doripenem C_{max} of 2.5 mg/liter with colistin (0.5 or 2 mg/liter) generally resulted in ~4- to 5-$\log_{10}$ greater killing across 24 to 72 h compared to results with monotherapy, whereas both combinations containing a doripenem C_{max} of 25 mg/liter generally resulted in ~2- to 3-$\log_{10}$ greater killing across the same period; all combinations resulted in sustained periods where no viable bacteria were detected. Of the 12 cases from 24 to 72 h (i.e., 4 combinations across 3 time points), 11 were synergistic, with the remaining case being additive (Fig. 1B and Table 3). Though activity was reduced somewhat at the higher inoculum with combinations containing a doripenem C_{max} of 2.5 mg/liter, both combinations containing a doripenem C_{max} of 25 mg/liter substantially increased activity and resulted in ~5- to 7-$\log_{10}$-greater killing at most time points across 24 to 72 h (Fig. 2B and Table 3). The latter combinations resulted in undetectable bacterial counts on at least two occasions and, for the combination of colistin at 2 mg/liter plus a doripenem C_{max} of 25 mg/liter, no viable bacteria from 48 h onwards. Bacterial killing was similarly enhanced for reference strain ATCC 13883. Although the combinations of colistin at 0.5 or 2 mg/liter plus a doripenem C_{max} of 25 mg/liter failed to substantially improve activity at the low inoculum (owing to the potent bacterial killing of equivalent doripenem monotherapy), the remaining two combinations at the low inoculum and all combinations at the high inoculum substantially increased bacterial killing over the corresponding monotherapy. Notably, at the high inoculum, all combinations produced bacterial killing to below the limit of detection by 26 to 28 h (~3- to 4-$\log_{10}$-greater killing than with doripenem monotherapy with a C_{max} of 25 mg/liter), with no viable bacteria detected after this time for all combinations except that of colistin at 0.5 mg/liter plus a doripenem C_{max} of 2.5 mg/liter; with the latter combination, regrowth to ~2-$\log_{10}$ CFU/ml occurred by 72 h, which was >3-$\log_{10}$ CFU/ml lower than that with the most active equivalent monotherapy (doripenem).

For colistin-resistant isolate KP035 at the 10^6-CFU/ml inoculum, only the combination of colistin at 2 mg/liter plus a doripenem C_{max} of 25 mg/liter resulted in enhanced bacterial killing. This combination provided additional killing of ~3- and 6.5-$\log_{10}$ CFU/ml at 24 and 72 h, respectively, over that achieved with doripenem monotherapy; from 50 h onwards, no viable bacteria were detected with this combination (Fig. 1D and Table 3). At the high inoculum, bacterial killing and regrowth with combination therapy were very similar to those produced with equivalent doripenem monotherapy (Fig. 2D and Table 3).

Emergence of colistin resistance. Apart from a small shift to the right at the 10^6-CFU/ml inoculum, PAPs for the growth controls at 72 h were generally similar to those observed at baseline. For the colistin-susceptible isolate (KP032), monotherapy with colistin at 0.5 mg/liter at the 10^6 CFU/ml inoculum resulted in an increase in colistin-resistant subpopulations by 24 h, growing in the presence of colistin at 10 mg/liter at 72 h; with higher colistin regimens (2 and 5 mg/liter), the low regrowth observed across 72 h at this inoculum precludes meaningful interpretation of the PAPs (Fig. 1C). At the high inoculum, a small number of resistant colonies emerged by 24 h with the colistin 0.5-mg/liter regimen, with the proportion of resistant colonies remaining approximately the same up to 72 h. At this inoculum, the colistin 5-mg/liter regimen resulted in a rapid and substantial increase in colistin-resistant colonies such that by 24 h, virtually all colonies grew in the presence of colistin at 10 mg/liter; the colistin 2-mg/liter regimen led to a slower but substantial emergence of colistin-resistant subpopulations across 72 h (Fig. 2C). For the two heteroresistant strains, all colistin monotherapy regimens (0.5, 2, and 5 mg/liter) resulted in substantial increases in colistin-resistant subpopulations by 24 to 48 h. At the 10^6-CFU/ml inoculum, all colistin regimens led to emergence of resistance, with growth in the presence of 8 or 10 mg/liter colistin by 24 to 48 h (data not shown); at the high inoculum, all regimens resulted in a substantial increase in resistant subpopulations with colistin at 5 mg/liter at 72 h, resulting in nearly all subpopulations growing in the presence of colistin at 10 mg/liter on the PAP plates (Fig. 2A and B). For the colistin-resistant isolate KP035, the PAPs at baseline and across the 72-h incubation period did not change irrespective of inoculum, with virtually all colonies in the control and treatment groups growing in the presence of colistin at 10 mg/liter (Fig. 1D and 2D).

Combination therapy against the colistin-susceptible isolate KP032 resulted in the emergence of colistin-resistant subpopulations only with the combination of colistin at 0.5 mg/liter plus a doripenem C_{max} of 2.5 mg/liter and only at the high inoculum (Fig. 2C). Although many combinations at both inocula resulted in substantial bacterial killing, for KP032 no colistin-resistant colonies were detected at any time with the combination of colistin at 2 mg/liter and a doripenem C_{max} of 2.5 mg/liter at the 10^8-CFU/ml inoculum despite substantial regrowth (to ~6-$\log_{10}$ CFU/ml) at 72 h (Fig. 2C). For the colistin-heteroresistant strains, no colistin-resistant colonies were detected following the initiation of combination therapy at any time at either inoculum, even on the few occasions where regrowth was extensive, e.g., with isolate KP033 at the high inoculum, where regrowth to ~7-$\log_{10}$ CFU/ml occurred at 72 h with the combinations of colistin at 0.5 or 2 mg/liter plus a doripenem C_{max} of 2.5 mg/liter (Fig. 2B). Combination therapy had no effect on colistin resistance of the MDR colistin-resistant isolate.

DISCUSSION

With colistin (and polymyxin B) monotherapy, regrowth of *K. pneumoniae* as well as other bacterial species considered susceptible to colistin based upon MIC measurement is well documented both *in vitro* (3, 32, 44) and *in vivo* (25), even with concentrations well in excess of those which can be safely achieved clinically. Amplification of colistin-resistant subpopulations, which has been shown to contribute to the observed regrowth, may be par-

FIG 2 (Left) Time-kill curves with various clinically relevant dosage regimens of colistin (Col) and doripenem (Dor) alone and in combination at an inoculum of ~10^8 CFU/ml. (Right) PAPs at baseline and after 72 h of exposure to colistin monotherapy, colistin-doripenem combination therapy, or neither antibiotic (growth control). Doripenem monotherapy regimens were not included in colistin-PAPs examination. (A) ATCC 13883 (colistin heteroresistant, doripenem susceptible, MDR); (B) FADDI-KP033 (colistin heteroresistant, doripenem susceptible, MDR); (C) FADDI-KP032 (colistin susceptible, doripenem resistant, MDR); (D) FADDI-KP035 (colistin resistant, doripenem susceptible, MDR). The *y* axis starts at the limit of detection, and the limit of quantification is indicated by the dashed horizontal line.

ticularly important for *K. pneumoniae*, which has a high frequency of colistin heteroresistance (75% [37] and > 90% [44] of isolates in previous studies). With currently recommended CMS dosage regimens achieving low colistin concentrations in plasma (17, 43) and toxicity limiting dose escalation in many patients (11, 17), CMS monotherapy may be suboptimal. Consequently, the investigation of rational colistin combinations is essential. We systematically investigated the effectiveness of colistin alone and in combination with doripenem against a diverse array of *K. pneumoniae* isolates which included colistin-susceptible, -heteroresistant, and -resistant strains.

The dosage regimens of colistin and doripenem employed in the present study were carefully designed to reflect the plasma concentration-time profiles achieved in critically ill patients (17, 39, 43). Previously we have shown that both colistin and doripenem are almost entirely unbound in CAMHB (2, 5). Currently, there is no information on unbound plasma concentrations of colistin in humans. However, assuming plasma binding of colistin in patients is similar to that in animals (i.e., ~50% bound) (29, 52), the colistin dosage regimens of 0.5 and 2 mg/liter used in our combinations are indeed clinically achievable (17, 43). Colistin was administered as a constant infusion to mimic the flat concentration-versus-time profiles observed across a dosage interval at steady state (17, 43). All doripenem dosage regimens employed in the combinations are readily achieved in plasma after consideration of protein binding (5). Considering the inoculum effect of colistin and carbapenems (9, 10), experiments were conducted at both ~10^6 and ~10^8 CFU/ml; the latter inoculum mimics the high bacterial densities found in some infections (34, 47). In general, the colistin-and-doripenem combination maintained its excellent activity at both inocula (Table 3) and was less subject to an inoculum effect compared to monotherapy with either agent.

The PK/PD index that best predicts colistin activity against *K. pneumoniae* is currently unknown. Against *P. aeruginosa* (2, 12) and *A. baumannii* (13), area under the unbound drug concentration-time curve over 24 h in the steady state divided by the MIC (*f*AUC/MIC) is the index for colistin most closely correlated with bacterial killing. For *P. aeruginosa*, an *f*AUC/MIC of >~20 was required to achieve a 1-log reduction at 24 h against most isolates (2, 12); this value was somewhat lower (~10) for equivalent killing of *A. baumannii* (13). If an *f*AUC/MIC target of >20 is applied to the present study, only the colistin 0.5-mg/liter regimen failed to reach this value against colistin-susceptible and -heteroresistant isolates (*f*AUC/MIC of 12), whereas all regimens were considerably below a *f*AUC/MIC of 20 for the colistin-resistant isolate (Table 2). However, even with a *f*AUC/MIC of 48 (colistin 2-mg/liter regimen) or 120 (colistin 5-mg/liter regimen), monotherapy with colistin reduced bacterial growth by >1 log at 24 h only with the 10^6-CFU/ml inoculum (in five of six cases; Table 3). The PK/PD index most closely associated with doripenem activity against Gram-negative bacilli is the cumulative percentage of a 24-h period that the unbound drug concentration exceeds the MIC under steady-state PK conditions ($fT_{>MIC}$) (50). For doripenem, an $fT_{>MIC}$ of 35 to 45% is required for a >1-log reduction in bacterial growth (50). Against the doripenem-susceptible isolates, this target was achieved with all doripenem regimens; against the doripenem-resistant isolate, only the doripenem C_{max} 50-mg/liter regimen achieved this target (Table 2). While a >1-log bacterial kill at 24 h was achieved against the reference strain and all isolates at both inocula with doripenem C_{max} 25- and 50-mg/liter regimens, this was achieved in only half the cases across both inocula with the C_{max} 2.5-mg/liter regimen (Table 3). Combining colistin with doripenem produced substantially enhanced bacterial killing at 24 h in 20 out of 24 cases against colistin-susceptible and -heteroresistant isolates, even with a low index value for each drug. For example, with the colistin 0.5-mg/liter (*f*AUC/MIC of 12) plus doripenem C_{max} 2.5- or 25-mg/liter ($fT_{>MIC}$ < 35%) regimen against doripenem-resistant KP032, >1-log killing at 24 h occurred in 3 of 4 cases with the combination compared to 3 of 6 cases with equivalent monotherapy (Table 3).

Despite all isolates containing β-lactamases, improvements in bacterial killing over that of equivalent monotherapy were observed across the 72-h duration at both inocula against colistin-susceptible and -heteroresistant strains. Although enhanced bacterial killing was generally present with all combinations, the killing effect was particularly strong for combinations containing a doripenem C_{max} of 25 mg/liter with colistin dosage regimens of either 0.5 or 2 mg/liter. That the addition of a doripenem C_{max} of 25 mg/liter to low-dosage regimens of colistin (e.g., 0.5 mg/liter) can substantially improve bacterial killing is an important observation given that many patients will achieve only low plasma colistin concentrations with currently recommended CMS dosage regimens (17, 43). Encouragingly, improvements in activity with combination therapy (colistin at 2 mg/liter and a doripenem C_{max} of 25 mg/liter) were also observed against the colistin-resistant isolate, albeit only with the 10^6-CFU/ml inoculum.

Previous studies have utilized static time-kill methods to investigate the combination of a polymyxin (colistin or polymyxin B) and a carbapenem against *K. pneumoniae* (8, 16, 23, 41, 46, 49). While these studies generally reported enhanced activity (usually reported as synergy) with the combination, only a single, generally low inoculum (~10^6 CFU/ml) was employed, and experiments were conducted for no longer than 24 h. Only one study employed more than one dosage regimen of polymyxin B (41), and importantly, the emergence of polymyxin resistance (e.g., by using real-time PAPs) was not reported. To the best of our knowledge, the present study is the first to investigate polymyxin and carbapenem combination therapy against *K. pneumoniae* using an *in vitro* PK/PD model and to investigate the emergence of colistin resistance with combination therapy. On only one occasion were colistin-resistant colonies detected following combination therapy for the colistin-susceptible or -heteroresistant isolates, and this occurred at the high inoculum with the lowest-dosage regimens of colistin and doripenem (0.5 mg/liter and C_{max} at 2.5 mg/liter, respectively). Although the extensive killing generally observed against these isolates with the combination regimens complicates the interpretation of PAPs, on the few occasions where extensive regrowth (e.g., up to ~7-$\log_{10}$ CFU/ml) occurred with combination therapy, no colistin-resistant colonies were detected; the reason for the observed regrowth despite an apparent lack of colistin resistance is unknown and is under investigation. This important finding clearly suggests that combining doripenem with colistin against *K. pneumoniae* may reduce the emergence of colistin-resistant subpopulations.

As multidrug resistance increases and we await active new agents, rationally designed combinations of existing antibiotics are of paramount importance. We have shown for the first time that clinically relevant dosage regimens of colistin and doripenem in combination substantially increase bacterial killing against

colistin-susceptible, -heteroresistant (and doripenem-resistant), and colistin-resistant MDR *K. pneumoniae* and limit the emergence of colistin resistance. Further investigations of colistin combinations in animal models and patients are warranted.

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