Synergistic Killing of Multidrug-Resistant *Pseudomonas aeruginosa* at Multiple Inocula by Colistin Combined with Doripenem in an *In Vitro* Pharmacokinetic/Pharmacodynamic Model

Phillip J. Bergen, Brian T. Tsuji, Jurgen B. Bulitta, Alan Forrest, Jovan Jacob, Hanna E. Sidjabat, David L. Paterson, Roger L. Nation, and Jian Li

Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Melbourne, Australia; School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, SUNY, Buffalo, New York; Ordway Research Institute, Albany, New York; and University of Queensland Centre for Clinical Research, Royal Brisbane and Women’s Hospital, Brisbane, Australia

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Combination therapy may be required for multidrug-resistant (MDR) *Pseudomonas aeruginosa*. The aim of this study was to systematically investigate bacterial killing and emergence of colistin resistance with colistin and doripenem combinations against MDR *P. aeruginosa*. Studies were conducted in a one-compartment *in vitro* pharmacokinetic/pharmacodynamic model for 96 h at two inocula (~10⁶ and ~10⁸ CFU/ml) against a colistin-heteroresistant reference strain (ATCC 27853) and a colistin-resistant MDR clinical isolate (19147 n/m). Four combinations utilizing clinically achievable concentrations were investigated. Microbiological response was examined by log changes and population analysis profiles. Colistin (constant concentrations of 0.5 or 2 mg/liter) plus doripenem (peaks of 2.5 or 25 mg/liter every 8 h; half-life, 1.5 h) substantially increased bacterial killing against both strains at the low inoculum, while combinations containing colistin at 2 mg/liter increased activity against ATCC 27853 at the high inoculum; only colistin at 0.5 mg/liter plus doripenem at 2.5 mg/liter failed to improve activity against 19147 n/m at the high inoculum. Combinations were additive or synergistic against ATCC 27853 in 16 and 11 of 20 cases (4 combinations across 5 sample points) at the 10⁶- and 10⁸-CFU/ml inocula, respectively; the corresponding values for 19147 n/m were 16 and 9. Combinations containing doripenem at 25 mg/liter resulted in eradication of 19147 n/m at the low inoculum and substantial reductions in regrowth (including to below the limit of detection at ~50 h) at the high inoculum. Emergence of colistin-resistant subpopulations of ATCC 27853 was substantially reduced and delayed with combination therapy. This investigation provides important information for optimization of colistin-doripenem combinations.

Multidrug-resistant (MDR) *Pseudomonas aeruginosa* is one of several important Gram-negative bacteria emerging as significant pathogens worldwide (8, 50). With a very limited number of therapeutic options against these pathogens remaining and a lack of novel antimicrobial agents in the drug development pipeline (30, 50), particularly those with activity against *P. aeruginosa* (50), clinicians have been forced to reexamine the use of “old,” previously discarded drugs such as the polymyxins (8, 40). Colistin (also known as polymyxin E) is a multicomponent cationic polypeptide antibiotic largely abandoned in the 1970s due to concerns about the potential for nephro- and neurotoxicity (15, 27). Colistin retains significant *in vitro* activity against Gram-negative “superbugs” and is often the only therapeutic option available to treat infections caused by these pathogens (1, 27, 35). Several institutions have already experienced outbreaks of infections with MDR Gram-negative bacteria resistant to all commercially available antibiotics except the polymyxins (6, 26, 34). Of particular concern is that with the rapid increase in the use of colistin over the last decade, especially for critically ill patients (8, 27), has come an increase in the number of reports of resistance to colistin (1, 23, 27).

Having entered clinical use in 1959, colistin was never subjected to the scientific rigor required for modern pharmaceuticals before they become available for use in patients. The result has been a dearth of reliable information on pharmacokinetics (PK) and pharmacodynamics (PD) with which to guide therapy, and confusion has surrounded the optimal dosing strategy. It is only very recently that crucial gaps in our knowledge of the PK and PD of colistin have begun to be filled. Recent investigations into the PK of colistin in critically ill patients have revealed low and potentially suboptimal plasma drug concentrations in a substantial proportion of patients receiving currently recommended dosage regimens (16, 47). In addition, both *in vitro* (3, 4, 48, 52) and *in vivo* (22, 32) studies have shown the potential for the rapid emergence of colistin resistance with monotherapy, with heteroresistance a likely contributing factor; colistin heteroresistance has been identified in *Acinetobacter baumannii* (28, 55), *Klebsiella pneumoniae* (48, 53), and most recently in *P. aeruginosa* (5a). The potential presence of colistin-resistant subpopulations of heteroresistant strains prior to therapy and the observation of rapid amplifi-
cation of colistin-resistant subpopulations with colistin monotherapy suggest caution with the use of colistin monotherapy and highlight the importance of investigating rational and novel colistin combinations. The aim of the present study was to systematically investigate the extent of in vitro bacterial killing and the emergence of colistin resistance with colistin alone and in combination with doripenem at both high and low inocula of *P. aeruginosa* using clinically relevant dosage regimens. This was achieved by simulating, in an in vitro PK/PD model, the PK of colistin formation and doripenem in humans over a range of clinically achievable concentrations in critically ill patients.

(Parts of this study were presented at the 50th Interscience Conference on Antimicrobial Agents and Chemistry [ICAAC], Boston, MA, 12 to 15 September 2010.)

**MATERIALS AND METHODS**

**Bacterial isolates.** Two strains of *P. aeruginosa* were employed in this study: a colistin-heteroresistant reference strain, ATCC 27853 (American Type Culture Collection, Rockville, MD), and a nonmucoid colistin-resistant MDR clinical isolate, strain 19147 n/m, obtained from a patient with cystic fibrosis; the clinical isolate was preserved at −80°C in cryovials (Sermint Plastics, Belcoill, Quebec, Canada).

**Antibiotics and reagents.** For MIC determinations and in vitro PK/PD studies, colistin sulfate (lot 109K1574; 23,251 U/mg) was purchased from Sigma-Aldrich (St. Louis, MO), while doripenem (lot 0137Y01) was kindly donated by Johnson (Shionogi and Co., Osaka, Japan). Colistin sulfate was used in the present study as colistin is the active antibacterial agent formed after administration of its inactive prodrug, colistin methanesulfonate (CMS) (5). Doripenem unbound in CAMHB (f_u) is almost entirely unbound in CAMHB, the PK/PD model and colistin-doripenem dosing regimens.

**In vitro PK/PD model and colistin-doripenem dosing regimens.** Experiments to examine the microbiological response and emergence of resistance to various dosage regimens of colistin and doripenem alone and in combination were conducted over 96 h at two different starting inocula (10^6 and 10^8 CFU/ml) using a one-compartment in vitro PK/PD model described previously (4) and below. Prior to each experiment, strains were subcultured onto horse blood agar (Media Preparation Unit, The University of Melbourne, Parkville, Australia) and incubated at 35°C for 24 h. One colony was then selected and grown overnight in 10 ml of CAMHB, from which early-log-phase broth was inoculated into each compartment of the experiment to yield ~10^8 CFU/ml. To achieve a starting inoculum of 10^6 CFU/ml, the flow of medium was temporarily halted, a 1.0-ml aliquot of overnight culture was inoculated into each compartment on the morning of the experiment, and the bacteria were allowed to grow until 10^8 CFU/ml was obtained. The experiment was commenced immediately upon attainment of 10^6 CFU/ml. The PK/PD model consisted of eight sealed containers (compartments) each containing 80 ml of CAMHB at 37°C and a magnetic stir bar to ensure adequate mixing. One compartment acted as a control to define growth dynamics in the absence of antibiotic, while colistin and/or doripenem was delivered into the remaining compartments to achieve the desired concentration (for colistin) or intermittent (doripenem) dosage regimen (see below). A peristaltic pump (Masterflex L/S; Cole-Parmer) was used to deliver sterile CAMHB from separate central reservoirs into each compartment at a predetermined rate, delivering an equal volume of CAMHB into a waste reservoir at the completion of the experiment to yield 10^8 CFU/ml. For colistin-containing regimens, colistin was delivered as a constant concentration by spiking colistin into the central reservoir prior to initiation of the experiment so that all media flowing through the system (with the exception of that in the growth control compartment) contained a constant concentration of colistin (Table 1); colistin was administered in this way to enable flat plasma concentration-time profiles. For doripenem-containing regimens, doripenem at steady state was observed in critically ill patients given CMS (16, 47). For colistin-containing regimens at the higher inoculum (~10^8 CFU/ml), each compartment was initially filled with sterile drug-free CAMHB to allow bacterial growth up to 10^6 CFU/ml in the absence of the drug; subsequently, a loading dose of colistin was administered to attain the targeted colistin concentration. For doripenem-containing regimens, doripenem was injected into each treatment compartment following bacterial inoculation to achieve the desired steady-state maximum (peak) concentration (C_max), with intermittent 8-hourly dosing thereafter (Table 1); as doripenem does not accumulate following multiple intravenous (i.v.) administrations, no loading dose was required to achieve steady-state concentrations. The chosen flow rate simulated a doripenem elimination half-life (t_1/2) of 1.5 h, which approximates that in critically ill patients (33). Three constant concentrations of colistin and three intermittent doripenem dosage regimens were simulated for monotherapy (Table 1). For colistin, the chosen flow rate resulted in a concentration-time profile with a rapid increase (t_max), with intermittent 8-hourly dosing there after (Table 1).

**Microbiological response and emergence of resistance to colistin.** Serial samples (0.6 ml) were collected aseptically at the times shown in Table 1 from each reservoir for viable-cell counting and real-time PAPs, as well as determination of colistin and doripenem concentrations. Viable-cell counts and PAPs were obtained.

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Concentrations of 0.5, 2.0, and 5.0 mg/liter, respectively.

PhenoSphere-NEXT 5-

10,000 rpm for 10 min. An aliquot of the sample (50

log_{10} CFU per milliliter from 0 h (CFU 0) to time

therapy were examined using the log change method, calculating the change in

concentration exceeds the MIC under steady-state PK conditions.

Active. We considered synergy to be a

\[ \text{additivity was defined as a 1- to} \]

f_3 - ( 3 + \text{morpholino)propanesulfonic acid (MOPS) buffer and 400 µl of meth-

anol were added, and the mixture was subjected to a vortex and centrifuged at

10,000 rpm for 10 min. An aliquot of the sample (50 µl) was injected onto a

Phenosphere-NEXT 5-µm C_{18} column (250 by 4.6 mm; Phenomenex, Torrance,

CA). A gradient elution procedure involving 100% methanol and 0.1% trifluo-

roacetic acid as the mobile phases was used, the proportion of methanol increas-

ing from 5 to 80% over 4 min and then returning to 5% over 0.5 min; the flow

rate was 0.7 ml/min, with detection at 311 nm. The run time was 10 min. The

assay for doripenem was 0.5 to 32 mg/liter; samples were diluted when the expec-
ted doripenem concentrations were higher than the upper limit of quantifi-
cation. Analysis of quality control (QC) samples with nominal concentrations of

0.40 and 4.0 mg/liter for colistin and 1.2, 12, and 48 mg/liter for doripenem (the

latter QC sample requiring dilution) demonstrated accuracy of >90% and co-
efficients of variation of <10.2% for both colistin and doripenem.

PD analysis. Microbiological responses to monotherapy and combination

treatment were examined using the log change method, calculating the change in

log_{10} CFU per milliliter from 0 h (CFU 0) to time t (6, 24, 48, 72 or 96 h; CFU 0)
as shown: log change = \log_{10}(\text{CFU} t) - \log_{10}(\text{CFU} 0).

Single-antibiotic or combination regimens causing a reduction of ≥1 log_{10}

CFU/ml below the initial inoculum at 6, 24, 48, 72, or 96 h were considered

active. We considered synergy to be a ≥2-log_{10}-lower number of CFU per

milliliter for the combination than for its most active component at the specified
time (46); additivity was defined as a 1- to <2-log_{10}-lower number of CFU per

milliliter for the combination.

RESULTS

PK validation and doripenem binding. The colistin drug

concentrations achieved (means ± standard deviations [SD])

were 0.45 ± 0.07 mg/liter (n = 22), 1.76 ± 0.17 mg/liter (n = 26),

and 4.58 ± 0.02 mg/liter (n = 6) for the targeted

cconcentrations of 0.5, 2.0, and 5.0 mg/liter, respectively.

Measured doripenem C_{\text{max}} and minimum (trough) concen-
tration (C_{\text{min}}) values were 51.47 ± 3.96 mg/liter (n = 30)

and 1.24 ± 0.42 mg/liter (n = 30) for the targeted values of

50.0 and 1.24 mg/liter and 25.60 ± 2.53 mg/liter (n = 50) and

0.80 ± 0.26 mg/liter (n = 50) for the targeted values of 25.0

and 0.62 mg/liter. For the targeted doripenem C_{\text{max}} of 2.5

mg/liter, the measured C_{\text{max}} was 2.45 ± 0.32 mg/liter (n = 50),

with all C_{\text{max}} values below the limit of quantification (0.5 mg/
liter) of the HPLC assay. Typical simulated PK profiles for
doripenem dosage regimens of 25 and 50 mg/liter every 8 h are shown in Fig. 1.
The observed mean t_{1/2} for the simulated intermittent doripenem dosage regimens was 1.55 ± 0.17 h (n = 71) for the targeted value of 1.5 h; as the C_{\text{min}} for some dosage regimens was below the lower limit of quantification of the HPLC assay, t_{1/2} was not directly measured in all experiments. The f_u at equilibrium was 0.95, indicating practical equivalence of total and unbound concentrations.

Microbiological response. The initial inocula (means ± SD)

were 6.20 ± 0.10 log_{10} CFU/ml (n = 11) and 8.09 ± 0.08 log_{10}

CFU/ml (n = 11) for ATCC 27853 and 6.30 ± 0.16 log_{10}

FIG. 1. Targeted doripenem (Dor) PK profiles for 25- and 50-mg/liter 8-hourly regimens with measured Dor concentrations.

TABLE 1. Colistin and doripenem dosage regimens, PK/PD index values, and sampling times in the in vitro PK/PD model a

<table>
<thead>
<tr>
<th>Treatment regimen</th>
<th>Target C_{\text{max}}/C_{\text{min}} (mg/liter)</th>
<th>Value for ATCC 27853/initial 19147 n/ml</th>
<th>Sampling times (h) for microbiological measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col monotherapy b</td>
<td>0.5</td>
<td>12.0/0.09</td>
<td>0, 1, 2, 3, 4, 6, 23, 24, 25, 26, 47, 48, 49, 50, 71, 72, 73, 74, 75, 96</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>48.0/0.38</td>
<td>100/100</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>120.0/0.94</td>
<td>100/100</td>
</tr>
<tr>
<td>Dor monotherapy c</td>
<td>2.5/0.062</td>
<td>158.6/3.3</td>
<td>2.5/10</td>
</tr>
<tr>
<td></td>
<td>25/0.62</td>
<td>158/633</td>
<td>25/100</td>
</tr>
<tr>
<td></td>
<td>50/1.24</td>
<td>317/1.266</td>
<td>50/200</td>
</tr>
<tr>
<td>Combination therapy d</td>
<td>0, 1, 2, 3, 4, 8, 23, 24, 25, 26, 29, 32, 47, 48, 49, 50, 53, 56, 71, 72, 73, 74, 77, 80, 95, 96</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Dosage regimens were tested with −10⁰ and −10⁰ CFU/ml starting inocula.

b Colistin (Col) dosage regimens involved a constant concentration of colistin simulating continuous infusion. For the colistin-resistant isolate (19147 n/ml), only colistin at 5.0 mg/liter was used as monotherapy. Values shown for isolate 19147 n/ml at other dosages of colistin are those for combination therapy with the indicated concentration of colistin.

c Doripenem (Dor) dosage regimens involved intermittent administration (every 8 h) to achieve the targeted C_{\text{max}}/C_{\text{min}}.

Microbiological response.

\[ \text{Microbiological response. The initial inocula (means ± SD)} \]

were 6.20 ± 0.10 log_{10} CFU/ml (n = 11) and 8.09 ± 0.08 log_{10}

CFU/ml (n = 11) for ATCC 27853 and 6.30 ± 0.16 log_{10}

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FIG. 1. Targeted doripenem (Dor) PK profiles for 25- and 50-mg/liter 8-hourly regimens with measured Dor concentrations.
CFU/ml \((n = 9)\) and 7.88 ± 0.28 log_{10} CFU/ml \((n = 9)\) for
19147 n/m for the targets of 10^6 and 10^8 CFU/ml, respectively. The
time course profiles of bacterial numbers achieved with all
dosage regimens at both inocula are shown in Fig. 2 (for ATCC
27853) and Fig. 3 (for 19147 n/m). Log changes in viable-cell
counts at each inoculum with mono- and combination therapy
are presented in Table 2.

**Colistin monotherapy.** With ATCC 27853 at the 10^6-
CFU/ml inoculum, colistin monotherapy produced rapid and
extensive initial killing at all concentrations, with colistin at 2
and 5 mg/liter resulting in undetectable bacterial counts at 2 h
(Fig. 2A). Substantial regrowth was evident at 6 h with colistin
at 0.5 mg/liter and at 24 h with colistin at 2 mg/liter, with
regrowth approaching that of the control by 24 h (for 0.5
mg/liter) and 72 h (for 2 mg/liter). No viable colonies were
detected until 54 h with colistin at 5 mg/liter, with subsequent
regrowth to \(-4\) log_{10} CFU/ml observed at 96 h. An inoculum
effect with colistin monotherapy was observed, with substan-
tially reduced initial bacterial killing at the high compared to
the low inoculum with colistin at 0.5 and 2 mg/liter (Fig. 2D).
While rapid and extensive initial bacterial killing to below
the limit of detection remained at the high inoculum with colistin
at 5 mg/liter, substantial regrowth (to \(-3.5\) log_{10} CFU/ml) had
occurred by 6 h, with regrowth to above the level of the initial
inoculum by 30 h. For the colistin-resistant isolate, bacterial growth in the presence of colistin at 5 mg/liter was essentially no different from that of the control with either inoculum (Fig. 3A and C).

**Doripenem monotherapy.** With ATCC 27853 at the 10^6-CFU/ml inoculum, all doripenem regimens (2.5, 25, or 50 mg/liter every 8 h) produced initial bacterial killing of ~2.5 log₁₀ CFU/ml, with regrowth beginning by 6 h (Fig. 2B). Regrowth close to control levels had occurred by 48, 72, and 96 h with concentrations of 2.5, 25, and 50 mg/liter, respectively. At the high inoculum, all doripenem concentrations produced similar killing profiles, with the 2.5-mg/liter 8-hourly regimen resulting in bacterial counts consistently ~0.5 to 1 log below control values and the 25- and 50-mg/liter regimens yielding bacterial counts ~1.5 to 3 log below control values (Fig. 2E). With the MDR isolate, doripenem at 2.5 mg/liter every 8 h produced only minimal bacterial killing (~1- to 2-log₁₀ reduction in CFU/ml) at each inoculum, with regrowth close to control values by 24 to 48 h (Fig. 3A and C). Higher doripenem concentrations (25 and 50 mg/liter) produced rapid initial killing of ~3 log at 6 h, with subsequent regrowth to within ~1 log of control values at 96 h (Fig. 3A and C). No inoculum effect was observed with doripenem against either strain.

**Combination therapy.** With ATCC 27853, the addition of doripenem at 2.5 or 25 mg/liter to colistin at 0.5 mg/liter produced an initial (i.e., up to 8-h) period of additional bacterial killing of ~2.5 log₁₀ CFU/ml compared with the most active monotherapy (colistin) at the low inoculum and resulted in undetectable bacterial counts no later than 3 h (Table 2). Both combinations resulted in synergy or additivity at most time points across 96 h (Table 2). Synergy was particularly evident with the combination of colistin at 0.5 mg/liter and doripenem at 2.5 mg/liter, with ~3- to 4-log₁₀-greater killing at most time points. Nevertheless, by 96 h regrowth with this regimen approached that of the growth control. The addition of doripenem (2.5 or 25 mg/liter) to colistin at 2 mg/liter produced synergy at 48 and 72 h, and this combination remained additive at 96 h with regrowth close to the level of the initial inoculum (Fig. 2C and Table 2). At the high inoculum, combinations of colistin at 0.5 mg/liter and doripenem (2.5 or 25 mg/liter) produced only modest increases in bacterial killing across the first 8 to 24 h, with regrowth thereafter similar to that in the presence of the most active single agent (doripenem) (Fig. 2F). With combinations containing colistin at 2 mg/liter, rapid and substantial reductions in bacterial counts were observed, with additional killing of ~3.5 log₁₀ CFU/ml over that achieved with the most active monotherapy at 8 h for the combination with doripenem at 2.5 mg/liter and additional killing of ~5 log₁₀ CFU/ml achieved at 4 h for the combination with doripenem at 25 mg/liter; with the latter combination, no viable bacteria were detected at 4 h. Synergy or additivity was maintained with these combinations across 48 and 96 h with doripenem at 2.5 and 25 mg/liter, respectively (Table 2).
Against ATCC 27853 at the 10^5 CFU/ml inoculum, colistin at 0.5 mg/liter plus doripenem at 2.5 mg/liter produced synergy at 24 and 48 h, with regrowth approaching control values by 72 to 96 h (Fig. 3B and Table 2). A similar killing profile was generated with the combination of colistin at 2 mg/liter and doripenem at 2.5 mg/liter, although initial bacterial killing was greater (by ~7.5 logs) and lower bacterial counts were maintained across the first 60 h (Fig. 3B). With the latter regimen, bacterial counts as low as 1.6 log_{10} CFU/ml (at 29 h) were observed. With combinations containing colistin (0.5 or 2 mg/liter) and doripenem at 2.5 mg/liter, the initial rate and extent of killing up to 4 to 6 h were similar to those with doripenem monotherapy (Fig. 3B). By 8 and 24 h, no viable bacteria were observed with the combinations containing colistin at 2 and 0.5 mg/liter, respectively, and no regrowth was subsequently detected. At the high inoculum, the combination of colistin at 0.5 mg/liter and doripenem at 2.5 mg/liter was essentially inactive (Fig. 3D). Increasing the concentration of colistin to 2 mg/liter produced greater bacterial killing at both 24 h (additive) and 48 h (synergistic), with regrowth to control levels by 72 h (Fig. 3D and Table 2). Substantially greater killing was observed with combinations containing doripenem at 25 mg/liter. The addition of doripenem at 25 mg/liter to colistin (0.5 or 2 mg/liter) produced substantial reductions in log_{10} CFU/ml compared to the equivalent doripenem monotherapy by 8 h (with colistin at 2 mg/liter) and 29 h (with colistin at 0.5 mg/liter) (Fig. 3D). No viable bacteria were detected at ~50 h with both combinations, with regrowth at 96 h substantially below (by ~3.5 to 5 log_{10} CFU/ml) that occurring with equivalent doripenem monotherapy (Fig. 3D).

**Emergence of colistin resistance.** Apart from a small shift to the right from 0 to 96 h at the 10^5 CFU/ml inoculum, the PAPs for ATCC 27853 at 96 h closely matched those observed at baseline at both inocula. With this strain, a small number of colistin-resistant colonies were detected at baseline at the high inoculum and for both inocula following 96 h of incubation in the model (Table 3). Colistin at 0.5 or 2 mg/liter resulted in substantial increases in the proportion of colistin-resistant subpopulations at both inocula (Fig. 4 and Table 3). With colistin at 5 mg/liter, the substantially lower growth at 96 h (~4.3 log_{10} CFU/ml) using an initial inoculum of 10^6 CFU/ml makes comparison of the PAPs at this time point difficult. However, at the 10^7 CFU/ml inoculum, a substantial increase in colistin-resistant subpopulations was evident by 24 h with 5-mg/liter colistin monotherapy (Fig. 3 and Table 3). For 19147 n/m, the PAPs at baseline and across the 96 h of incubation did not change, irrespective of the inoculum or colistin treatment (data not shown).

**Combination therapy against ATCC 27853** substantially reduced the emergence of colistin-resistant subpopulations (Table 3). When doripenem at 2.5 mg/liter was added to colistin (0.5 or 2 mg/liter) at both inocula, a small shift to the right in the PAPs from 72 to 96 h was generally observed (Fig. 4). The emergence of colistin-resistant subpopulations at both inocula

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**Table 2. Log changes in CFU/ml**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Inoculum (CFU/ml)</th>
<th>Time (h)</th>
<th>Log change [log_{10}(CFU_0) - log_{10}(CFU_t)]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Col 0.5 mg/L Col 2 mg/L Col 5 mg/L Dor 2.5 mg/L Dor 25 mg/L Dor 50 mg/L Col 0.5 mg/L + Dor 2.5 mg/L Col 0.5 mg/L + Dor 25 mg/L Col 2 mg/L + Dor 2.5 mg/L</td>
</tr>
<tr>
<td>ATCC 27853</td>
<td>10^6</td>
<td>6</td>
<td>-1.71</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24</td>
<td>1.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>1.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
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<tr>
<td></td>
<td></td>
<td>96</td>
<td>1.20</td>
</tr>
<tr>
<td>19147 n/m</td>
<td>10^6</td>
<td>6</td>
<td>-0.36</td>
</tr>
<tr>
<td></td>
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<td>24</td>
<td>-0.42</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>-0.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
<td>-0.73</td>
</tr>
<tr>
<td></td>
<td></td>
<td>96</td>
<td>-0.82</td>
</tr>
<tr>
<td>19147 n/m</td>
<td>10^6</td>
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<tr>
<td></td>
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<td>96</td>
<td>-0.82</td>
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* Log changes in CFU/ml at 6, 24, 48, 72, and 96 h at an inoculum of 10^6 or 10^8 CFU/ml with colistin (Col) and/or doripenem (Dor) against *P. aeruginosa*. The gray background indicates activity (a reduction of ≥1-log_{10} CFU/ml below the initial inoculum); the green background indicates synergy (a ≥2-log_{10} decrease in the number of CFU per milliliter with the combination compared to its most active component); the red background indicates additivity (a 1.0- to <2-log_{10} decrease in the number of CFU per milliliter with the combination compared to its most active component). ††, colistin-heteroresistant reference strain. Heteroresistance to colistin was defined as the ability of subpopulations of a strain to grow in the presence of ≥2 mg/liter although the colistin MIC for the strain was ≤2 mg/liter. ‡‡, nonmucoid MDR colistin-resistant clinical isolate (colistin monotherapy was performed with 5 mg/liter only).
was suppressed even further with the addition of doripenem at 25 mg/liter to colistin (0.5 or 2 mg/liter) (Fig. 4). For example, with a starting inoculum of 10⁶ CFU/ml, the combination of colistin at 2 mg/liter plus doripenem at 2.5 mg/liter resulted in substantially fewer colonies growing in the presence of ≥4 mg/liter colistin at 96 h than the equivalent colistin monotherapy (Fig. 4F). The number of resistant colonies was reduced even further with the combination of colistin at 0.5 mg/liter plus doripenem at 25 mg/liter, despite similar levels of growth at this time point with all three regimens. Combination therapy had no effect on colistin resistance of the MDR colistin-resistant isolate (data not shown).

**DISCUSSION**

Colistin is increasingly used as salvage therapy in critically ill patients for otherwise untreatable MDR infections (15, 27). However, regrowth of colistin-susceptible *P. aeruginosa* with colistin (or polymyxin B) monotherapy is commonly observed (4, 10, 18, 22, 51), even with colistin concentrations well above those which can be safely achieved clinically. In addition, recent population PK studies employing currently recommended CMS dosage regimens indicate that the plasma colistin concentrations achieved in critically ill patients are in many cases suboptimal (16, 47). Given the potential for the rapid emergence of colistin resistance with monotherapy, combination therapy against *P. aeruginosa* has been suggested as a possible means by which to increase antimicrobial activity and reduce the development of resistance (29). We systematically investigated the effectiveness of colistin alone and in combination with doripenem against a colistin-heteroresistant strain and an MDR colistin-resistant isolate of *P. aeruginosa*. Doripenem was chosen because of its high potency against MDR *P. aeruginosa* (11, 38) and its low potential for selection of carbapenem-resistant *P. aeruginosa* (19, 37, 49). As some data show that the activity of colistin (10) and carbapenems alone (36) is attenuated at high compared to low inocula, in the present study experiments were conducted at both ~10⁶ and ~10⁷ CFU/ml; the latter inoculum mimics the high bacterial densities found in some infections.

The dosage regimens of colistin and doripenem used in the present study were carefully chosen to reflect the plasma drug concentration-time profiles achieved in critically ill patients. Intravenous administration of CMS, the parenteral formulation of colistin, results in average steady-state plasma colistin concentrations of ~2 to 3 mg/liter, with some patients achieving concentrations up to ~10 mg/liter (16, 31, 47). As colistin concentrations at steady state remain more or less constant (16, 47), colistin was administered as a constant infusion. We have previously demonstrated that colistin is almost entirely unbound in CAMHB (3). Thus, the colistin concentrations of 0.5 and 2 mg/liter used in our study are clinically achievable, assuming plasma binding of colistin in patients is similar to that in animals (i.e., ~50% is bound) (25). Unfortunately, although the knowledge of total plasma colistin concentrations achieved in patients is increasing, there is currently no information on unbound plasma colistin concentrations in humans. Though the majority of PK data on doripenem have been obtained with healthy volunteers, plasma drug concentration-time profiles for patients appear to be similar to those for healthy volunteers (39). Doripenem is typically administered intermittently every 8 h, with a standard 500-mg dose achieving a Cmax of ~25 mg/liter (7, 42). As binding of doripenem in the growth medium was minimal, all doripenem concentrations employed in the combinations are readily achieved in plasma after consideration of protein binding (7, 14, 20, 42).

To our knowledge, this is the first study to investigate the combination of colistin plus doripenem against *P. aeruginosa* using an *in vitro* PD model and to utilize colistin PK data recently obtained from critically ill patients (discussed below). An inoculum effect was generally observed for colistin monotherapy, whereas no obvious inoculum effect was present for doripenem (Fig. 2 and 3). The addition of doripenem to colistin resulted in substantial improvements in bacterial killing over equivalent monotherapy against the MDR colistin-resistant isolate at both inocula, particularly with a doripenem

<table>
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<th>Inoculum (CFU/ml)</th>
<th>Time (h)</th>
<th>Control</th>
<th>Col at 0.5 mg/liter</th>
<th>Col at 2 mg/liter</th>
<th>Col at 5 mg/liter</th>
<th>Col at 0.5 mg/liter + Dor at 2.5 mg/liter</th>
<th>Col at 0.5 mg/liter + Dor at 25 mg/liter</th>
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⁴ ND, no colistin-resistant subpopulations detected.
concentration of 25 mg/liter. Though the benefits in overall antibacterial activity with the combination were slightly less pronounced against the colistin-susceptible but -heteroresistant strain, combination regimens nevertheless resulted in substantial improvements in bacterial killing, particularly with combinations containing colistin at 2 mg/liter. Overall, our data suggest that the addition of doripenem to even low concentrations of colistin (e.g., 0.5 mg/liter) can substantially improve antibacterial activity. Given the current last-line status of colistin therapy, we have reported not only synergy but also additivity, as even a relatively small increase in activity with clinically achievable concentrations of both antibiotics may be beneficial to patient care.

Previous studies employing static time-kill methods have examined colistin in combination with a carbapenem (imipenem, meropenem, or doripenem) against *P. aeruginosa*, with mixed results (2, 5a, 12, 43, 44). In these previous studies, investigations were undertaken for no longer than 48 h (usually 24 h) with a single dose of each antibiotic administered at the commencement of treatment. Of these studies, only our previous study employed multiple inocula and investigated the emergence of colistin resistance (5a); that study included both
isolates used in the present study. While concentrations of antibiotics in that study and the present study are not directly comparable and the former study examined colistin in combination with imipenem, the activities of colistin combined with either imipenem or doripenem were similar across 48 h (the duration of the former study) at both inocula of ATCC 27853. However, substantial differences against the MDR colistin-resistant isolate were evident. In the static model, combinations with concentrations as high as 32 mg/liter colistin plus imipenem at 16× MIC failed to reduce bacterial numbers to below the limit of detection at any time point. In stark contrast, bacterial eradication was achieved in the PK/PD model with combinations containing colistin (0.5 or 2 mg/liter) and doripenem at 25 mg/liter no later than 24 h at the low inoculum, and bacteria were reduced to below detectable levels at approximately 48 h with the same combinations at the high inoculum. This highlights the importance of simulating PK profiles when examining PD responses.

Though *P. aeruginosa* can undergo adaptive resistance to polymyxins (17), the report of colistin heteroresistance in *P. aeruginosa* (5a) and changes in PAPs following treatment with colistin monotherapy (4, 5a, 10) suggest that amplification of preexisting colistin-resistant subpopulations is a contributing factor in the regrowth observed with colistin monotherapy. This pattern was similarly observed in the present study with colistin monotherapy. Though the meaningful interpretation of PAPs is difficult when combination therapy has led to extensive killing, an important finding of the present study is that when bacterial numbers were comparable (within ~1 to 2 log_{10} CFU/ml of those achieved with the equivalent monotherapy), combination therapy against the colistin-heteroresistant strain at both inocula substantially reduced and delayed the emergence of colistin-resistant subpopulations. Whereas colistin-resistant colonies emerged rapidly (often within 24 h) with colistin monotherapy, with combination therapy resistant colonies generally emerged later (following 72 to 96 h of treatment) and formed a substantially smaller proportion of the overall bacterial population (Table 3). In addition, the most resistant subpopulations (i.e., those growing in the presence of colistin at 10 mg/liter on the PAP plates) were absent with combination therapy. In contrast, we previously reported that changes in the PAPs with colistin and imipenem combination therapy in a static time-kill model generally mirrored those observed with equivalent exposure to colistin monotherapy (5a). Loss of imipenem due to degradation in the static experiments likely contributed to this result (21). Intermittent dosing of doripenem in the present study replenishes doripenem concentrations and avoids the combination’s effectively becoming colistin monotherapy over time. This reported difference highlights once again the importance of PK/PD models in assessing the activity of and the emergence of resistance to antimicrobial therapy.

We have previously suggested two possible reasons for an enhanced PD effect observed with the combination of colistin and a carbapenem (9). Subpopulation synergy involves one drug killing the subpopulation(s) resistant to the other drug and *vice versa*. ATCC 27853 is colistin heteroresistant, indicating the existence of colistin-resistant subpopulations prior to therapy. Though regrowth of this strain occurred with all combinations, it was considerably reduced with combinations containing each drug at the higher concentration, particularly over the first 48 to 72 h. Interestingly, high-level colistin resistance did not emerge despite the regrowth. While subpopulation synergy may have contributed to an enhanced PD effect against this isolate, it cannot explain the substantially enhanced activity of colistin-doripenem combinations against the MDR colistin-resistant isolate given its near complete resistance to colistin (MIC, 128 mg/liter). This enhanced activity occurred despite the presence of enzymes active against carbapenems. Mechanistic synergy involves colistin and doripenem acting on different cellular pathways to increase the rate or extent of killing by the other drug. It is possible that permeabilization of the outer membrane by colistin (56) resulted in substantially increased concentrations of doripenem in the periplasm, allowing greater access to the critical penicillin-binding proteins located on the cytoplasmic membrane where the carbapenems act (41, 54). Subpopulation and mechanistic synergies are not mutually exclusive, and both may operate simultaneously. Further investigations are ongoing to elucidate the mechanism(s) underpinning the enhanced PD activity observed.

We have shown for the first time that clinically relevant dosage regimens of colistin and doripenem in combination substantially increase killing of both colistin-susceptible (and -heteroresistant) and MDR colistin-resistant *P. aeruginosa*, even at a high initial inoculum. Combination therapy also substantially reduced and delayed the emergence of colistin resistance. Our data highlight the importance of prospective optimization of colistin combinations using a translational PK/PD approach. Further investigations of colistin combinations in animal infection models and patients are warranted to optimize colistin-doripenem combinations targeting isolates which are resistant to all antibiotics, including the last-line therapy colistin.

**ACKNOWLEDGMENTS**

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**REFERENCES**

in combination with imipenem enhance pharmacodynamic activity against multidrug-resistant Pseudomonas aeruginosa at multiple inocula. Anti-
30. Bulitta, J. B., et al. 2010. Attenuation of colistin bactericidal activity by high inoculum of Pseudomonas aeruginosa characterized by a new mechanism-
31. Caskey, M. R., N. N. Jones, and D. M. Livermore. 2009. Antimicrobial activities of doripenem and other carbapenems against Pseudomonas aerugi-
34. Crandon, J. L., C. C. Bulik, and D. P. Nicolau. 2009. In vivo efficacy of 1- and 2-gram human simulated prolonged infusions of doripenem against Pseu-
anesulfonate and formed colistin in critically ill patients from a multicenter study provide dosing suggestions for various categories of patients. Antimi-
37. Gilleland, H. E., J. R. Chalmip, and R. S. Conrad. 1984. Chemical alterations in cell envelopes of Pseudomonas aeruginosa upon exposure to polymyxin: a possible mechanism to explain adaptability to poly-
38. Gundersen, B. W., et al. 2003. Synergistic activity of colistin and ceftazidime against multidrug-resistant Pseudomonas aeruginosa in an in vitro pharc-
41. Keel, R. A., C. A. Sutherland, L. J. Crandon, and D. P. Nicolau. 2011. Stability of doripenem, imipenem and meropenem at elevated room tem-
44. Li, J., et al. 2001. A simple method for the assay of colistin in human plasma, using pre-column derivatization with 9-fluorenylmethyl chloro-
formate in solid-phase extraction cartridges and reversed-phase high-
45. Li, J., et al. 2003. Use of high-performance liquid chromatography to study the pharmacokinetics of colistin sulfate in rats following intravenous admin-
48. Li, J., et al. 2006. In vitro resistance to colistin in multidrug-resistant Acin-
49. Lister, P. D., D. J. Wolter, and N. D. Hanson. 2009. Antibacterial-resistant Pseudomonas aeruginosa: clinical impact and complex regulation of chro-
51. Markou, N., et al. 2008. Colistin serum concentrations after intravenous administration in critically ill patients with serious multidrug-resistant, gram-
negative infections: the use of colistin. Rev. Exp. Anti. Infection Ther. 8:1009–
1017.

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