AAC Accepts, published online ahead of print on 12 September 2011
Antimicrob. Agents Chemother. doi:10.1128/AAC.05298-11

Synergistic killing of multidrug-resistant *Pseudomonas aeruginosa* at multiple inocula by colistin combined with doripenem in an *in vitro* PK/PD model

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Running title: Colistin and doripenem against *Pseudomonas aeruginosa* in an *in vitro* PK/PD model

Key words: colistin, doripenem, combination, *Pseudomonas aeruginosa*

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

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Abstract

Combination therapy may be required for 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* PK/PD model for 96 h at two inocula (~10^6 and ~10^8 cfu/mL) against a colistin-heteroresistant reference strain (ATCC 27853) and 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/L) plus doripenem (peaks of 2.5 or 25 mg/L eight hourly, half-life 1.5 h) substantially increased bacterial killing against both strains at the low inoculum, while combinations containing colistin 2 mg/L increased activity against ATCC 27853 at the high inoculum; only colistin 0.5 mg/L plus doripenem 2.5 mg/L 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 10^6 and 10^8 inocula, respectively; the corresponding values for 19147 n/m were 16 and 9. Combinations containing doripenem 25 mg/L resulted in bacterial 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 in ATCC 27853 was substantially reduced and delayed with combination therapy. This investigation provides important information for optimization of colistin/doripenem combinations.

Introduction

Multidrug-resistant *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 remaining against these pathogens, and a lack of novel antimicrobial agents in the drug development pipeline (31, 50), particularly those with activity against *P. aeruginosa* (50), clinicians have been forced to re-examine the use of 'old', previously discarded drugs such as the polymyxins (8, 41). Colistin (also known as polymyxin E) is a multi-component cationic polypeptide antibiotic largely abandoned in the 1970s due to concerns about the potential for nephro- and neuro-toxicity (16, 28). 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, 28, 36). Several institutions have already experienced outbreaks of multidrug-resistant (MDR) Gram-negative bacteria resistant to all commercially available antibiotics except the polymyxins (6, 27, 35). Of particular concern is that with the rapid increase in use of colistin over the last decade, especially in critically-ill patients (8, 28), has come a concomitant increase in the number of reports of resistance to colistin (1, 24, 28).

Having entered clinical use in 1959, colistin was never subjected to the scientific rigour required of modern pharmaceuticals before they become available for use in patients. The result has been a dearth of reliable pharmacokinetic (PK) and pharmacodynamic (PD) information 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 sub-optimal plasma concentrations in a substantial proportion of patients receiving currently recommended dosage regimens (17, 47). In

addition, both *in vitro* (3-4, 48, 52) and *in vivo* (23, 33) 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* (29, 55), *Klebsiella pneumoniae* (48, 53), and most recently in *P. aeruginosa* (manuscript submitted). The potential presence of colistin-resistant subpopulations prior to therapy in heteroresistant strains, and the observation of rapid amplification of colistin-resistant subpopulations with colistin monotherapy, suggests caution with the use of colistin monotherapy and highlights 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 emergence of colistin resistance with colistin alone and in combination with doripenem at both high and low inocula against *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.

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, USA), and a non-mucoid colistin-resistant multidrug-resistant (MDR) clinical isolate, 19147 n/m, obtained from a patient with cystic fibrosis; the clinical isolate contained genes encoding IMP type carbapenemase and CTX-M type extended-spectrum β-lactamase (ESBL). Heteroresistance to colistin was defined as an isolate with a colistin minimum inhibitory concentration (MIC) ≤2 mg/L in which subpopulations were able to grow in the presence of >2 mg/L colistin in the population analysis profiles (PAPs; see below). MDR was defined as diminished

susceptibility to ≥2 of the following five drug classes: antipseudomonal cephalosporins, antipseudomonal carbapenems, β-lactam-β-lactamase inhibitor combinations, antipseudomonal fluoroquinolones, and aminoglycosides (45). MICs of colistin (sulphate) and doripenem were each 1 mg/L for ATCC 27853, and 128 mg/L and 0.25 mg/L for 19147 n/m, respectively. MICs to colistin and doripenem for each isolate were determined in three replicates on separate days in cation-adjusted Mueller-Hinton broth (CAMHB, Ca^{2+} at 23.0 μg/mL, Mg^{2+} at 12.2 μg/mL; Oxoid, Hampshire, England) via broth microdilution (13). Resistance to colistin (13) and doripenem (15) was defined as MIC ≥4 mg/L. Strains were stored in tryptone soy broth (Oxoid, Basingstoke, Hampshire, England) with 20% glycerol (Ajax Finechem, Seven Hills, New South Wales, Australia) at –80ºC in cryovials (Simport Plastics, Boloeil, Quebec, Canada).

Antibiotics and reagents

For MIC determinations and *in vitro* PK/PD studies, colistin sulphate was purchased from Sigma-Aldrich (lot 109K1574, 23,251 units/mg; St Louis, MO), while doripenem was kindly donated by Johnson and Johnson (lot 0137Y01; Shionogi and Co, Osaka, Japan). Colistin sulfate was used in the current study as colistin is the active antibacterial agent formed *in vivo* after administration of its inactive prodrug, colistin methanesulfonate (CMS) (5). Stock solutions of doripenem were prepared using Milli-Q water (Millipore Australia, North Ryde, New South Wales, Australia) immediately prior to each dose and protected from light to minimize loss from degradation, then sterilized by filtration with a 0.22-μm-pore-size Millex-GP filter (Millipore, Bedford, MA). Colistin was similarly prepared at the beginning of each experiment and spiked into the growth media of the central reservoir (see below) to achieve the desired concentration; preliminary experiments demonstrated colistin was stable under

these conditions for the duration of the experiment. All other chemicals were from suppliers previously described (25).

Binding of doripenem in growth medium

The binding of doripenem in CAMHB was measured by equilibrium dialysis using Dianorm equilibrium dialyzer units containing two chambers (1 mL in each chamber) separated by a semipermeable membrane (regenerated cellulose membrane, molecular weight cut-off 10k Daltons; Harvard Apparatus, Holliston, MA). Doripenem was spiked into CAMHB (donor chamber) to achieve a concentration of 25 mg/L and dialyzed at 37˚C against the same volume of isotonic phosphate buffer pH 7.4 (acceptor chamber); samples were prepared in triplicate. Samples of CAMHB and buffer were removed from each reservoir after 4 h (shown in preliminary studies to be the time required for equilibration) and stored at –80˚C until analyzed as described below. The fraction of doripenem unbound in CAMHB (f_u) was calculated as follows: (acceptor doripenem concentration)/(donor doripenem concentration).

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 35ºC for 24 h. One colony was then selected and grown overnight in 10 mL of CAMHB, from which early log-phase growth was obtained. For a starting inoculum of ~10^6 cfu/mL, a 1.0-mL aliquot of this early-log-phase bacterial suspension was inoculated into each compartment at the commencement of the experiment to

yield ~10^6 cfu/mL. To achieve a starting inoculum of ~10^8 cfu/mL, flow of media was temporarily halted and a 1.0-mL aliquot of overnight culture inoculated into each compartment on the morning of the experiment and allowed to grow until 10^8 cfu/mL was obtained. The experiment was commenced immediately upon attainment of 10^8 cfu/mL.

The PK/PD model consisted of eight sealed containers (compartments) each containing 80 mL of CAMHB at 37ºC 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 were delivered into the remaining compartments to achieve the desired constant concentration (colistin) or intermittent (doripenem) dosage regimens (see below). A peristaltic pump (Masterflex L/S, Cole-Parmer, USA) was used to deliver sterile CAMHB from separate central reservoirs into each compartment at a predetermined rate, displacing an equal volume of CAMHB into a waste receptacle. Flow rates were calibrated prior to each experiment and monitored throughout to ensure the system was performing optimally. 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 the growth control compartment) contained a constant concentration of colistin (Table 1); colistin was administered in this way to mimic the flat plasma concentration-time profiles of formed colistin at steady-state observed in critically-ill patients administered CMS (17, 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 10^8 cfu/mL in the absence of drug; subsequently, a loading dose of colistin was administered to immediately 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

peak concentration (C_{max}), with intermittent 8-hourly dosing thereafter (Table 1); as doripenem does not accumulate following multiple IV administration 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 (34).

Three constant concentration colistin and three intermittent doripenem dosage regimens were simulated for monotherapy (Table 1). For combination therapy against both isolates, colistin at a constant concentration of 0.5 or 2.0 mg/L was used in combination with intermittent doripenem at concentrations of 2.5 or 25 mg/L, yielding four combination regimens (Table 1); combination dosage regimens mimicked the PK profiles of each drug achieved in critically-ill patients (17, 32, 47). As we have previously demonstrated that colistin (3), and in the present study doripenem, are almost entirely unbound in CAMHB, the specified concentrations represent unbound (free) concentrations.

Microbiological response and the emergence of resistance to colistin

Serial samples (0.6 mL) were collected aseptically at 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 counting and PAPs were conducted immediately after sampling by spiral plating (WASP2 spiral plater, Don Whitley Scientific Ltd, UK) 50 µL of appropriately diluted sample (using 0.9% saline) onto either nutrient agar (viable counting) or Mueller-Hinton agar (PAPs), followed by incubation at 35ºC for 24 h (48 h for plates with small colonies). Serial dilutions and plating with the spiral plater, which further dilutes the sample, helped reduce the possibility of antibiotic carryover. PAPs plates were impregnated with colistin (sulphate) at 0, 0.5, 1, 2, 3, 4, 5, 6, 8 and 10 mg/L; these concentrations were chosen after consideration of the MICs and the colistin concentrations typically achievable in

plasma after intravenous CMS administration in patients (17, 32, 47). Full PAPs incorporating all colistin concentrations were determined at 0 and 96 h; mini-PAPs (0, 2, 4 and 8 mg/L) were determined at 6, 24, 48, and 72 h. Colonies were counted using a ProtoCOL colony counter (Don Whitley Scientific Ltd, UK); the limit of detection was 20 cfu/mL (equivalent to 1 colony per plate), and limit of quantification 400 cfu/mL (equivalent to 20 colonies per plate), as specified in the ProtoCOL manual.

Pharmacokinetic validation

Samples (100 µL) collected in duplicate from the *in vitro* PK/PD experiments were placed in 1.5 mL microcentrifuge tubes (Greiner Bio-One, Frickenhausen, Germany) and immediately stored at -80ºC until analysis; all samples were assayed within 4 weeks. Concentrations of colistin were measured using high-performance liquid chromatography (HPLC) (26) with an assay range for colistin sulfate of 0.10 to 6.00 mg/L**.** Doripenem concentrations were assayed at ambient temperature using a validated reversed-phase HPLC method. The HPLC system consisted of a Shimadzu LC-20AD Prominence liquid chromatograph, SIL-20AC HT Prominence autosampler and SPD-M20A Prominence diode array detector (Shimadzu, Columbia, MD, USA). To 100 µL of sample, 100 µL of 3-(N-morpholino)propanesulfonic acid (MOPS) buffer and 400 µL of methanol were added, vortexed and centrifuged at 10,000 rpm for 10 min. An aliquot of the sample (50 µL) was injected onto a Phenosphere-NEXT 5 µ C18 column (250 mm × 4.6 mm; Phenomenex, Torrance, California, USA). A gradient elution procedure involving 100% methanol and 0.1% trifluoroacetic acid as the mobile phases was used, the proportion of methanol increasing from 5% to 80% over 4 min 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 range for doripenem was 0.5 to 32 mg/L; samples were diluted when the expected doripenem concentrations were higher than the upper limit of

quantification. Analysis of quality control (QC) samples with nominal concentrations of 0.40 and 4.0 mg/L for colistin and 1.2, 12, and 48 mg/L for doripenem (the latter QC sample requiring dilution) demonstrated accuracy of >90% and coefficients of variation <10.2% for both colistin and doripenem.

Pharmacodynamic analysis

Microbiological response to monotherapy and combination therapy was examined using the log change method comparing the change in $\log_{10}$ (cfu/mL) from 0 h (CFU_0) to time t (6, 24, 48, 72 or 96 h; CFU_t) as shown:

$$\text{Log change} = \log_{10}(CFU_t) - \log_{10}(CFU_0)$$

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 cfu/mL for the combination relative to its most active component at the specified time (46); additivity was defined as a 1 to <2-$\log_{10}$ lower cfu/mL for the combination.

Results

Pharmacokinetic validation and doripenem binding

The colistin drug concentrations achieved (mean ± SD) were 0.45 ± 0.07 (n = 22), 1.76 ± 0.17 (n = 26) and 4.58 ± 0.02 (n = 6) mg/L for the targeted concentrations of 0.5, 2.0 and 5.0 mg/L, respectively. Measured doripenem C_{max} and trough concentration (C_{min}) concentrations were 51.47 ± 3.96 (n = 30) and 1.24 ± 0.42 (n = 30) mg/L for the targeted values of 50.0 and 1.24 mg/L, and 25.60 ± 2.53 (n = 50) and 0.80 ± 0.26 (n = 50) mg/L for the targeted values of 25.0 and 0.62 mg/L. For the targeted doripenem C_{max} of 2.5 mg/L, measured C_{max} concentrations were 2.45 ± 0.32 (n = 50), with all C_{min} concentrations below the limit of

quantification (0.5 mg/L) of the HPLC assay. Typical simulated PK profiles for doripenem dosage regimens of 25 and 50 mg/L 8-hourly are shown in Figure 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 C_{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 (mean ± SD) were 6.20 ± 0.10 (n = 11) and 8.09 ± 0.08 (n = 11) $\log_{10}$ cfu/mL for ATCC 27853, and 6.30 ± 0.16 (n = 9) and 7.88 ± 0.28 (n = 9) $\log_{10}$ cfu/mL 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 Figure 2 (ATCC 27853) and Figure 3 (19147 n/m). Log changes of viable cell counts at each inoculum with mono- and combination therapy are presented in Table 2.

Colistin monotherapy. Against ATCC 27853 at the 10^6 inoculum, colistin monotherapy produced rapid and extensive initial killing at all concentrations, with colistin 2 and 5 mg/L resulting in undetectable bacterial counts at 2 h (Fig. 2A). Substantial regrowth was evident at 6 h with colistin 0.5 mg/L and 24 h with colistin 2 mg/L, with regrowth approaching that of the control by 24 h (0.5 mg/L) and 72 h (2 mg/L). No viable colonies were detected until 54 h with colistin 5 mg/L, with subsequent regrowth to ~4 $\log_{10}$ cfu/mL observed at 96 h. An inoculum effect with colistin monotherapy was observed, with substantially reduced initial bacterial killing at the high compared to low inoculum with colistin 0.5 and 2 mg/L (Fig. 2D). While rapid and extensive initial bacterial killing to below the limit of detection remained at the high inoculum with colistin 5 mg/L, 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. Against the colistin-resistant isolate, bacterial growth in the presence of colistin 5 mg/L was essentially no different to that of the growth control at either inoculum (Fig. 3A and C).

Doripenem monotherapy. Against ATCC 27853 at the 10^6 inoculum, all doripenem regimens (2.5, 25 or 50 mg/L, 8-hourly) produced initial bacterial killing of ~2.5-$\log_{10}$ 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/L, respectively. At the high inoculum all doripenem concentrations produced a similar killing profile with the 2.5 mg/L 8-hourly regimen resulting in bacterial counts consistently ~0.5- to 1-log below control values, and 25 and 50 mg/L regimens bacterial counts ~1.5- to 3-log below control values (Fig. 2E). Against the MDR isolate, doripenem 2.5 mg/L 8-hourly produced only minimal bacterial killing (~1- to 2-$\log_{10}$ kill) 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/L) 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. Against ATCC 27853, the addition of doripenem 2.5 or 25 mg/L to colistin 0.5 mg/L produced an initial (i.e., up to 8 h) additional bacterial kill of ~2.5-$\log_{10}$ 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 0.5 mg/L and doripenem 2.5 mg/L, with ~3- to 4-$\log_{10}$ greater kill 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/L)

297 to colistin 2 mg/L produced synergy at 48 and 72 h, and remained additive at 96 h with
298 regrowth close to the level of the initial inoculum (Fig. 2C and Table 2). At the high
299 inoculum, combinations of colistin 0.5 mg/L and doripenem (2.5 or 25 mg/L) produced only
300 modest increases in bacterial killing across the first 8 to 24 h, with regrowth thereafter similar
301 to that of the most active single agent (doripenem) (Fig. 2F). With combinations containing
302 colistin 2 mg/L, rapid and substantial reductions in bacterial counts were observed with an
303 additional ~3.5 $\log_{10}$ cfu/mL kill over the most active monotherapy achieved at 8h with
304 doripenem 2.5 mg/L, and an additional ~5 $\log_{10}$ cfu/mL kill achieved at 4 h with doripenem
305 25 mg/L; with the latter combination, no viable bacteria were detected at this time. Synergy
306 or additivity was maintained with these combinations across 48 and 96 h with doripenem 2.5
307 and 25 mg/L, respectively (Table 2).
308
309 Against 19147 n/m at the 10^6 inoculum, colistin 0.5 mg/L plus doripenem 2.5 mg/L produced
310 synergy at 24 and 48 h, with regrowth approaching control values by 72 to 96 h (Fig. 3B and
311 Table 2). A similar killing profile was generated with the combination of colistin 2 mg/L and
312 doripenem 2.5 mg/L, although initial bacterial killing was greater (~3 log kill) and lower
313 bacterial counts maintained across the first ~60 h (Fig. 3B). With this latter regimen, bacterial
314 counts as low as 1.6 $\log_{10}$ cfu/mL (at 29 h) were observed. With combinations containing
315 colistin (0.5 or 2 mg/L) and doripenem 25 mg/L, the initial rate and extent of killing up to 4 –
316 6 h was similar to that of doripenem monotherapy (Fig. 3B). By 8 and 24 h, no viable
317 bacteria were observed with the combinations containing colistin 2 and 0.5 mg/L,
318 respectively, and no regrowth was subsequently detected. At the high inoculum, the
319 combination of colistin 0.5 mg/L and doripenem 2.5 mg/L was essentially inactive (Fig. 3D).
320 Increasing the concentration of colistin to 2 mg/L produced greater bacterial kill at both 24 h
321 (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 25 mg/L. The addition of doripenem 25 mg/L to colistin (0.5 or 2 mg/L) produced substantial reductions in $\log_{10}$ cfu/mL over that of equivalent doripenem monotherapy by 8 h (with colistin 2 mg/L) and 29 h (with colistin 0.5 mg/L) (Fig. 3D). No viable bacteria were detected at ~50 h with both combinations, with regrowth at 96 h substantially below (by ~3.5 – 5 $\log_{10}$ cfu/mL) that of equivalent doripenem monotherapy (Fig. 3D).

Emergence of colistin resistance

Apart from a small shift to the right from 0 to 96 h at the 10^6 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 incubation in the model (Table 3). Colistin 0.5 or 2 mg/L resulted in substantial increases in the proportion of colistin-resistant subpopulations at both inocula (Fig. 4 and Table 3). With colistin 5 mg/L, the substantially lower growth at 96 h (~4.3 $\log_{10}$ cfu/mL) using an initial inoculum of 10^6 makes comparison of the PAPs at this time difficult. However, at the 10^8 inoculum a substantial increase in colistin-resistant subpopulations was evident by 24 h with colistin 5 mg/L monotherapy (Fig. 3 and Table 3). For 19147 n/m, the PAPS at baseline and across the 96 h incubation period did not change irrespective of inoculum or colistin treatment (data not shown).

Combination therapy against ATCC 27853 substantially reduced the emergence of colistin-resistant subpopulations (Table 3). When doripenem 2.5 mg/L was added to colistin (0.5 or 2 mg/L) at both inocula, a small shift to the right of the PAPs was generally observed from 72 to 96 h (Fig. 4). The emergence of colistin-resistant subpopulations at both inocula was suppressed even further with the addition of doripenem 25 mg/L to colistin (0.5 or 2 mg/L)

(Fig. 4). For example, with a starting inoculum of 10^8 cfu/mL, the combination of colistin 2 mg/L plus doripenem 2.5 mg/L resulted in substantially fewer colonies growing in the presence of ≥4 mg/L colistin at 96 h compared with equivalent colistin monotherapy (Fig. 4F). The number of resistant colonies was reduced even further with the combination of colistin 0.5 mg/L plus doripenem 25 mg/L, despite a similar level of growth at this time 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 (16, 28). However, regrowth of colistin-susceptible *P. aeruginosa* with colistin (or polymyxin B) monotherapy is commonly observed (4, 10, 19, 23, 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 (17, 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 (30). We systematically investigated the effectiveness of colistin alone and in combination with doripenem against a colistin heteroresistant strain and a MDR-colistin-resistant isolate of *P. aeruginosa*. Doripenem was chosen because of its high potency against MDR *P. aeruginosa* (11, 39) and its low potential for selection of carbapenem-resistant *P. aeruginosa* (20, 38, 49). As some data show that activity of colistin (10) and carbapenems alone (37) is attenuated at high compared to low inocula, in the present study

experiments were conducted at both ~10^6 and ~10^8 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 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 – 3 mg/L, with some patients achieving concentrations up to ~10 mg/L (17, 32, 47). As colistin concentrations at steady-state remain more or less constant (17, 47), colistin was administered as a constant infusion. We have previously demonstrated that colistin is almost entirely unbound in CAMHB (3). Thus, colistin concentrations of 0.5 and 2 mg/L used in our study are clinically achievable, assuming plasma binding of colistin in patients is similar to that in animals (i.e. ~50% bound) (26). Unfortunately, although the knowledge of total plasma colistin concentrations achieved in patients is increasing, there is currently no information on unbound plasma concentrations in humans. Though the majority of PK data on doripenem has been obtained in healthy volunteers, plasma concentration-versus-time profiles in patients appear similar to those in healthy volunteers (40). Doripenem is typically administered intermittently every 8 h, with a standard 500 mg dose achieving a C_{max} of ~25 mg/L (7, 15). As binding of doripenem in the growth media was minimal, all doripenem concentrations employed in the combinations are readily achieved in plasma after consideration of protein binding (7, 14-15, 21).

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 utilise colistin PK data recently obtained from critically-ill patients (discussed subsequently). An inoculum effect was generally observed for colistin monotherapy, whereas no obvious inoculum effect was

present for doripenem (Figs.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 concentration of 25 mg/L. 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 2 mg/L. Overall, our data suggests that the addition of doripenem to even low concentrations of colistin (e.g., 0.5 mg/L) can substantially improve antibacterial activity. Given the current last-line status of colistin therapy, we 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, 12, 43-44, manuscript submitted). In these previous reports, 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 (manuscript submitted); that study included both isolates used in the present study. While concentrations of antibiotics between that and the present study are not directly comparable, and the former study examined colistin in combination with imipenem, the activity of colistin combined with either imipenem or doripenem was similar across 48 h (the duration of the former study) at both inocula against ATCC 27853. However, substantial differences were evident against the MDR-colistin-resistant isolate. In the static model, combinations with concentrations as high as 32 mg/L colistin plus 16× MIC imipenem failed to reduce bacterial numbers to below the

limit of detection at any time. In stark contrast, bacterial eradication was achieved in the PK/PD model with combinations containing colistin (0.5 or 2 mg/L) and doripenem 25 mg/L no later than 24 h at the low inoculum, and bacteria 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 (18), the report of colistin heteroresistance in *P. aeruginosa* (manuscript submitted), and changes in PAPs following treatment with colistin monotherapy (4, 10, manuscript submittted), suggest amplification of pre-existing colistin-resistant subpopulations is a contributing factor to the regrowth observed with colistin monotherapy. This was similarly observed in the present study with colistin monotherapy. Though the meaningful interpretation of PAPs is difficult where combination therapy has led to extensive killing, an important finding of the present study is that when bacterial numbers were comparable (within ~1-2 $\log_{10}$ cfu/mL of 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 10 mg/L on the PAPs plates) were absent with combination therapy. In contrast, we previously reported 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 (manuscript submitted). Loss of imipenem due to degradation in the static experiments likely contributed to this result (22).

Intermittent dosing of doripenem in the present study replenishes doripenem concentrations and avoids the combination effectively becoming colistin monotherapy over time. This reported difference highlights once again the importance of PK/PD models in assessing activity and emergence of resistance of 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 resistant subpopulation(s) of the other drug, and *vice versa*. ATCC 27853 is colistin-heteroresistant, indicating the existence of colistin-resistant subpopulations prior to therapy. Though regrowth occurred with this strain 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/L). 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 of the other drug. It is possible 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 (42, 54). Subpopulation and mechanistic synergy 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 bacterial killing against 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.

Acknowledgements

The project described was supported by Award Number R01AI079330 from the National Institute of Allergy and Infectious Diseases. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases or the National Institutes of Health. DLP has previously received honoraria from Merck for invited lectures and participation in advisory boards. JL is an Australian National Health and Medical Research Council Senior Research Fellow.

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680 **Table 1:** Colistin (Col) and doripenem (Dor) dosage regimens, PK/PD index values and

681 sampling times in the *in vitro* PK/PD model

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	Dosage regimens at ~10^6 and ~10^8 cfu/mL starting inocula						
	Col monotherapy[*†]			Dor monotherapy[‡]			Combination therapy
Target C_{max}/C_{min} (mg/L)	0.5	2.0	5.0	2.5/ 0.062	25/ 0.62	50/ 1.24	Col 0.5 + Dor 2.5 Col 0.5 + Dor 25 Col 2.0 + Dor 2.5 Col 2.0 + Dor 25
ATCC27853/ isolate 19147 n/m[§]							
AUC/MIC	12.0/ 0.09	48.0/ 0.38	120/ 0.94	15.8/ 63.3	158/ 633	317/ 1266	
C_{max}/MIC	0.5/ 0.004	2.0/ 0.02	5.0/ 0.04	2.5/ 10	25/ 100	50/ 200	
$\%T_{>MIC}$	0/0	100/ 0	100/ 0	24.8/ 62.3	87.1/ 100	100/ 100	
Sampling times (h) for microbiological measurements[**]	0, 1, 2, 3, 4, 6, 23, 24, 25, 26, 47, 48, 49, 50, 71, 72, 73, 74, 95, 96			0, 1, 2, 3, 4, 6, 23, 24, 25, 26, 30, 47, 48, 49, 50, 54, 71, 72, 73, 74, 78, 95, 96			0, 1, 2, 3, 4, 6, 8, 23, 24, 25, 26, 29, 32, 47, 48, 49, 50, 53, 56, 71, 72, 73, 74, 77, 80, 95, 96

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[*] Colistin dosage regimens involved a constant concentration of colistin simulating continuous infusion.

[†] For colistin-resistant isolate (19147 n/m), only Col 5.0 mg/L was used as monotherapy.

[‡] Doripenem dosage regimens involved intermittent administration 8-hourly to achieve the targeted C_{max}/C_{min}.

[§] Target values of PK/PD indices. For combination therapy, the values of the PK/PD indices for each drug are the same as for equivalent monotherapy.

[**] cfu/mL determined at all times. Full population analysis profiles (PAPs) were performed at 0 and 96 h; 'mini PAPs' were performed at 6, 24, 48 and 72 h.

Table 2: Log changes at 6, 24, 48, 72 or 96 h at an inoculum of 10^6 and 10^8 cfu/mL with colistin (Col) and/or doripenem (Dor) against *P. aeruginosa*. Gray background indicates activity (a reduction of ≥1-$\log_{10}$ cfu/mL below the initial inoculum); green background indicates synergy (a ≥2-$\log_{10}$ decrease in the number of cfu/mL between the combination and its most active component); 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).

Isolate	Inoculum (cfu/mL)	Time (h)	Log change (= $\log_{10}(CFU_t)$ - $\log_{10}(CFU_0)$)									
			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 + Dor 2.5	Col 0.5 + Dor 25	Col 2 + Dor 2.5	Col 2 + Dor 25
ATCC 27853[††]	10^6	6	-1.71	-6.18	-6.29	-1.55	-1.71	-2.36	**-6.27**	**-4.97**	-6.16	-6.04
		24	1.49	-2.96	-6.29	0.34	-0.34	-0.30	**-2.26**	**-1.37**	-2.27	**-4.57**
		48	1.12	-0.81	-6.29	1.20	0.43	-0.05	**-2.14**	-0.21	**-2.97**	**-3.35**
		72	1.41	0.69	-3.99	1.47	1.05	0.19	**-1.97**	**-0.67**	**-2.11**	**-2.20**
		96	1.20	1.10	-1.97	1.92	1.51	0.92	**0.07**	**-0.43**	**-0.14**	**-0.03**
	10^8	6	-0.36	-1.85	-4.69	-1.25	-2.79	-3.12	**-2.45**	**-3.82**	**-4.66**	**-8.17**
		24	-0.42	-1.53	-2.75	-1.17	-2.16	-2.54	**-2.97**	-2.68	**-3.53**	**-4.91**
		48	-0.75	-1.54	-1.91	-1.10	-2.07	-2.98	-1.32	-1.78	**-2.79**	**-5.83**
		72	-0.73	-1.50	-0.80	-0.74	-1.78	-1.86	-0.75	-1.82	-1.81	**-3.73**
		96	-0.82	-1.53	-0.79	-0.54	-1.66	-1.16	-0.88	-1.31	-1.53	**-3.67**
19147 n/m[‡‡]	10^6	6	-	-	0.83	-0.67	-2.39	-3.14	-1.42	-2.12	**-2.89**	**-3.58**
		24	-	-	1.47	-0.39	-3.26	-2.89	**-3.08**	**-6.52**	**-3.90**	**-6.22**
		48	-	-	1.21	1.28	-1.58	-0.45	**-1.45**	**-6.52**	**-2.09**	**-6.22**
		72	-	-	1.07	1.53	-0.63	0.21	**-0.08**	**-6.52**	**0.47**	**-6.22**
		96	-	-	0.47	1.69	-0.14	0.35	1.38	**-6.52**	1.59	**-6.22**
	10^8	6	-	-	0.01	-1.04	-2.79	-2.50	-0.95	-2.91	-1.82	**-4.32**
		24	-	-	-0.37	-0.58	-2.94	-1.96	-0.53	-3.67	**-2.42**	-2.73
		48	-	-	-0.59	-0.05	-1.37	-1.68	-0.51	**-8.06**	**-2.38**	**-3.47**
		72	-	-	-0.65	-0.04	-1.48	-1.28	-0.49	**-8.06**	-0.29	**-4.13**
		96	-	-	-0.99	-0.01	-1.35	-1.57	-0.69	**-6.46**	-0.27	**-4.24**

[††] colistin-heteroresistant reference strain; heteroresistance to colistin was defined as an isolate with colistin MIC ≤2 mg/L in which subpopulations were able to grow in the presence of >2 mg/L colistin

[‡‡] non-mucoid multidrug-resistant colistin-resistant clinical isolate; colistin monotherapy performed with 5 mg/L only

689

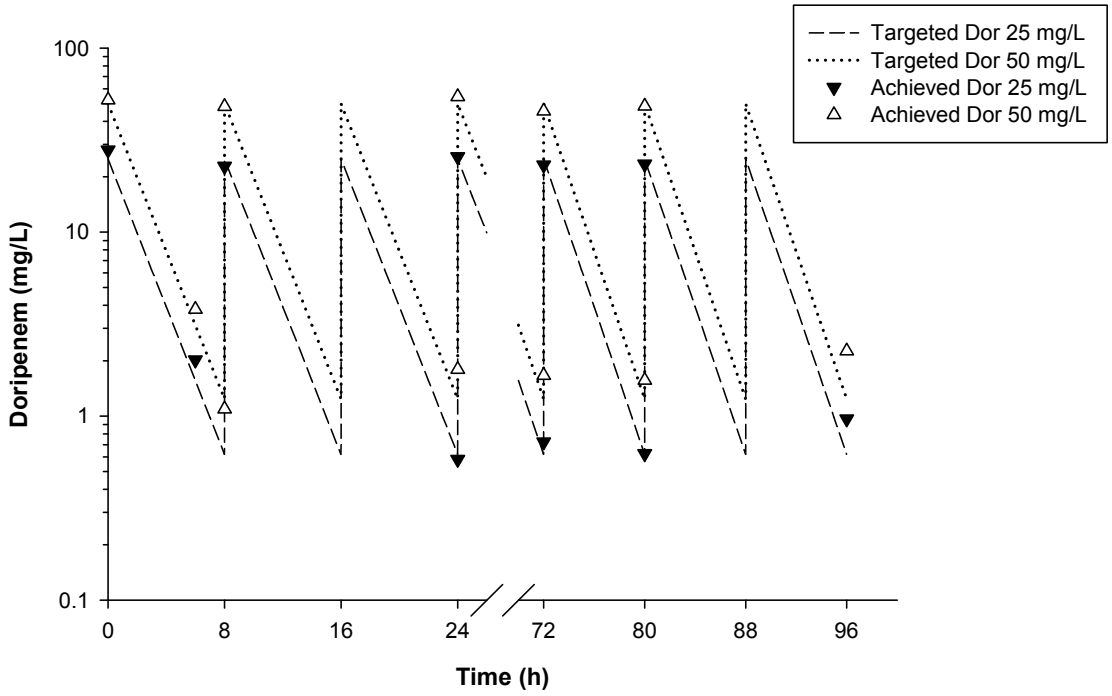
690 **Table 3:** Proportion of colistin-resistant subpopulations in *P. aeruginosa* ATCC 27853 at various times in the *in vitro* PK/PD model

Inoculum (cfu/mL)	Time (h)	Proportion of colistin-resistant subpopulations in the presence of 4 mg/L colistin							
		Control	Col 0.5 mg/L	Col 2 mg/L	Col 5 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	Col 2 mg/L + Dor 25 mg/L
10^6	0	ND[§§]	ND	ND	ND	ND	ND	ND	ND
	6	ND	ND	ND	ND	ND	ND	ND	ND
	24	ND	3.08×10^{-1}	ND	ND	ND	ND	ND	ND
	48	ND	2.82×10^{-1}	ND	ND	ND	ND	1.12×10^{-3}	ND
	72	ND	1.80×10^{-2}	8.58×10^{-3}	ND	ND	ND	2.67×10^{-3}	ND
	96	ND	3.67×10^{-2}	7.37×10^{-1}	ND	1.83×10^{-5}	ND	1.75×10^{-5}	8.78×10^{-5}
10^8	0	1.19×10^{-7}	1.72×10^{-7}	ND	4.05×10^{-7}	3.51×10^{-7}	9.60×10^{-7}	4.43×10^{-7}	7.38×10^{-6}
	6	5.81×10^{-8}	1.75×10^{-5}	3.67×10^{-5}	ND	ND	ND	ND	ND
	24	1.01×10^{-7}	8.22×10^{-6}	1.25×10^{-1}	1.29×10^{-2}	ND	ND	ND	ND
	48	1.83×10^{-7}	4.90×10^{-3}	2.65×10^{-1}	8.74×10^{-1}	3.70×10^{-6}	2.51×10^{-5}	4.57×10^{-5}	ND
	72	2.95×10^{-8}	3.18×10^{-3}	3.01×10^{-1}	9.49×10^{-1}	3.14×10^{-4}	ND	4.77×10^{-3}	ND
	96	8.92×10^{-8}	3.22×10^{-3}	2.72×10^{-1}	9.71×10^{-1}	7.78×10^{-4}	5.66×10^{-5}	6.55×10^{-3}	ND

691

692

[§§] ND: No colistin-resistant subpopulations detected.



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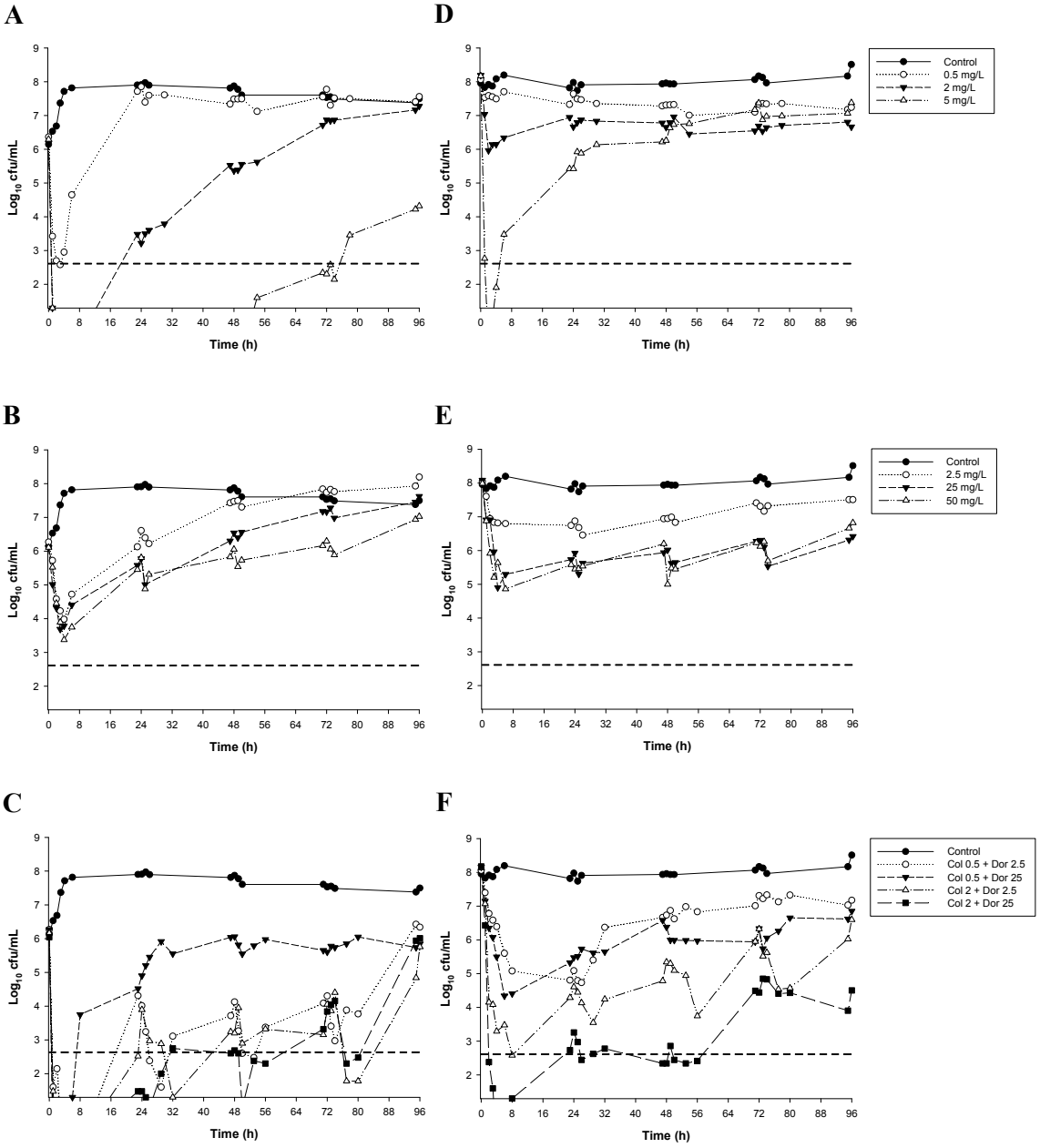
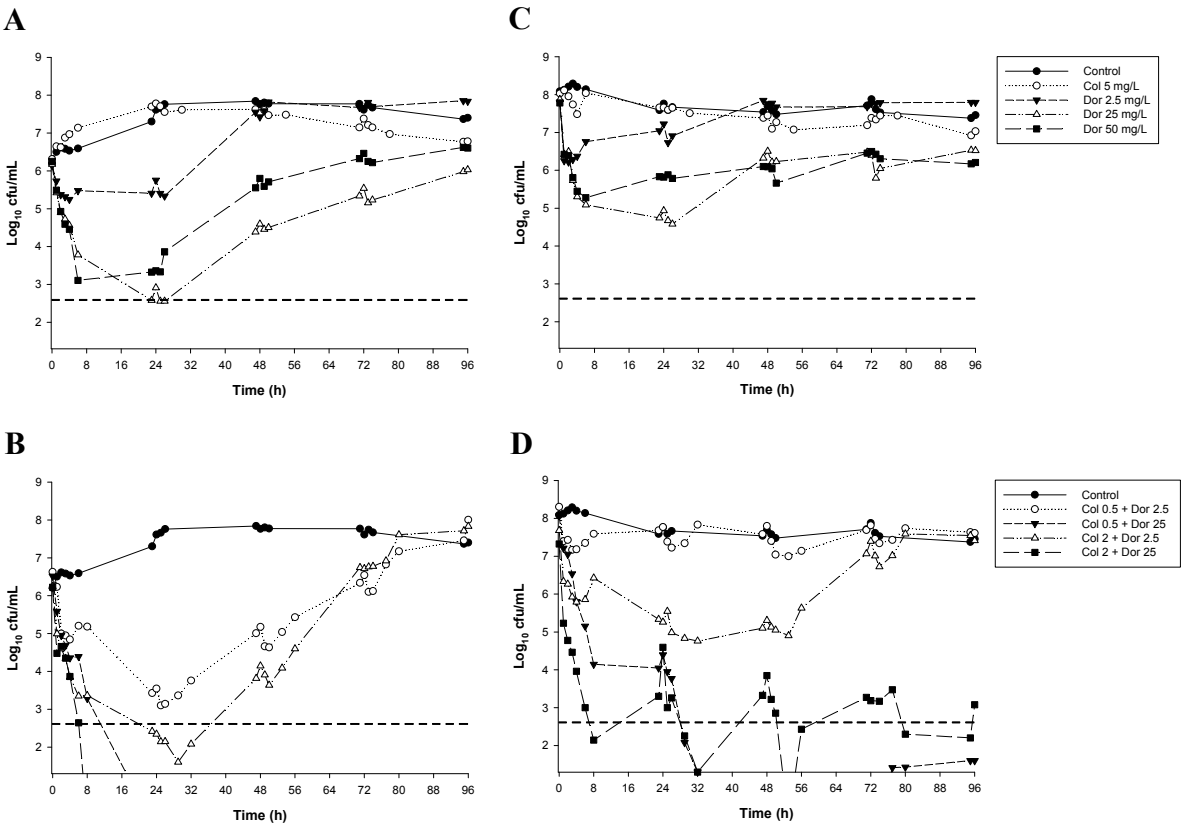
695

696 **Figure 1.** Targeted doripenem (Dor) pharmacokinetic profiles for 25 and 50 mg/L 8-hourly regimens with
697 measured Dor concentrations.



698

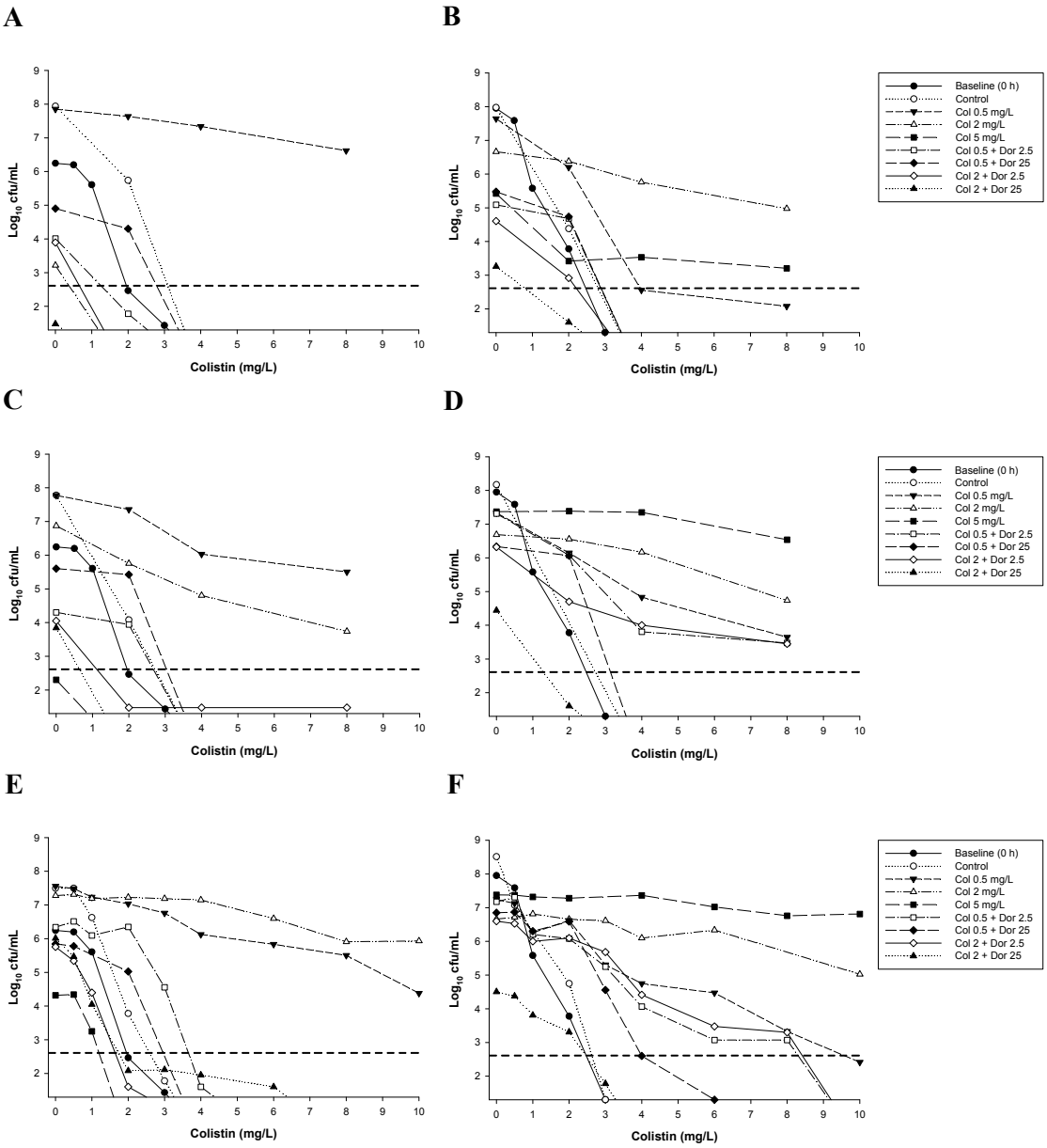
699 **Figure 2.** Time-kill curves for colistin monotherapy (Panels A and D), doripenem
700 monotherapy (Panels B and E) and the combination (Panels C and F) against ATCC 27853 at
701 the 10^6 cfu/mL (left-hand panels) and 10^8 cfu/mL (right-hand panels) inocula. The y-axis
702 starts from the limit of detection and the limit of quantification (LOQ) is indicated by the
703 horizontal broken line.



704

705 **Figure 3.** Time-kill curves for colistin and doripenem monotherapy (Panels A and C) and the
706 combination (Panels B and D) against 19147 n/m at 10^6 cfu/mL (left-hand panels) and 10^8
707 cfu/mL (right-hand panels) inocula. The y-axis starts from the limit of detection and the limit
708 of quantification (LOQ) is indicated by the horizontal broken line.

709



710

711 **Figure 4.** Population analysis profiles (PAPs) against ATCC 27853 with colistin
712 monotherapy, colistin plus doripenem combination therapy or neither antibiotic (control) at
713 10^6 cfu/mL inoculum (left-hand panels) and 10^8 cfu/mL inoculum (right-hand panels), at 24 h
714 (Panels A and B), 72 h (Panels C and D) and 96 h (Panels E and F). 0 h (baseline) PAPs are
715 shown in all panels. Colonies growing on ≥4 mg/L colistin are considered resistant. The y-
716 axis starts from the limit of detection and the limit of quantification (LOQ) is indicated by the
717 horizontal broken line.

718