Colistin and doripenem combinations against *Pseudomonas aeruginosa*: profiling the time course of synergistic killing and prevention of resistance

Neang S. Ly¹², Jürgen B. Bulitta¹³, Gauri G. Rao¹², Cornelia B. Landersdorfer¹³, Patricia N. Holden¹², Alan Forrest¹, Phillip J. Bergen⁴, Roger L. Nation³, Jian Li³ and Brian T. Tsuji¹²*

¹Laboratory for Antimicrobial and Bacterial Dynamics, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, State University of New York, Buffalo, NY, USA; ²The New York State Center of Excellence in Bioinformatics & Life Sciences, University at Buffalo, State University of New York, Buffalo, NY, USA; ³Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Monash University, Parkville, Victoria, Australia; ⁴Centre for Medicine Use and Safety, Monash University, Parkville, Victoria, Australia

*Corresponding author. Tel: +1-716-881-7543; Fax: +1-716-849-6890; E-mail: btsuji@buffalo.edu

Received 13 August 2014; returned 29 September 2014; revised 5 December 2014; accepted 21 December 2014

Objectives: Colistin is an ‘old’ drug, which is being increasingly utilized due to limited therapeutic options. However, resistance emergence during monotherapy is concerning. Here, our objective was to optimize colistin combinations against *Pseudomonas aeruginosa* by profiling the time course of synergistic killing and prevention of resistance.

Methods: Hollow-fibre infection models over 10 days simulated clinically relevant dosage regimens of colistin and doripenem against two heteroresistant *P. aeruginosa* strains (MIC 1 mg/L) and one resistant (MIC 128 mg/L) strain (inoculum 10⁹.³ cfu/mL). New mathematical mechanism-based models (MBMs) were developed using S-ADAPT.

Results: Against heteroresistant *P. aeruginosa* strains, colistin monotherapy resulted in initial killing (up to 2.64 log₁₀ cfu/mL) within 24 h followed by regrowth. High-intensity combinations involving free steady-state colistin concentrations of 5 mg/L achieved complete eradication (/>.9.3 log₁₀ killing) within 48 h. These combinations achieved synergy with up to 9.38 log₁₀ greater killing compared with the most active monotherapy. Against the colistin-resistant strain, the combination yielded marked initial synergy with up to 6.11 log₁₀ cfu/mL bacterial reductions within 72 h followed by regrowth. The MBMs quantified total and resistant subpopulations and the proposed synergy between colistin and doripenem.

Conclusions: Our findings provide insight into optimal antibiotic treatment and may serve as a framework for new drug combinations and combination modelling.

Keywords: polymyxin, combination therapy, *P. aeruginosa*, pharmacokinetics, pharmacodynamics

Introduction

MDR Gram-negative bacteria such as *Pseudomonas aeruginosa* are causing a global health crisis that is exacerbated by extremely limited treatment options.⁰¹ The severe lack of new antibiotics, especially against MDR Gram-negative bacteria, has led to the re-examination of ‘old’ antibiotics such as the polymyxins. Currently, polymyxin antibiotics (including polymyxin E, also called colistin) are commonly used empirically as a last line of defence against MDR Gram-negative pathogens, particularly for infections in critically ill patients.³

Although colistin monotherapy displays rapid and extensive killing in vitro against MDR *P. aeruginosa*-negative bacteria, rapid regrowth and the emergence of resistance in vitro and in patients is concerning.⁴⁵ This phenomenon is due in part to colistin heteroresistance, whereby pre-existing colistin-resistant subpopulations are amplified in isolates otherwise considered susceptible based on MIC determinations.⁶⁷ Recent clinical pharmacokinetic studies have demonstrated that, even with the currently recommended upper-limit daily dose, plasma concentrations of colistin are suboptimal in a significant proportion of critically ill patients.⁸⁹ Furthermore, colistin-induced nephrotoxicity can occur in up to 60% of patients receiving intravenous regimens and is a dose-limiting factor.¹⁰¹¹ Therefore, increasing the daily dose of colistin monotherapy is not a viable option to maximize bacterial killing and minimize the emergence of resistance in patients. While colistin combination therapies are an option, there are substantial gaps in knowledge of how to optimize such...
combinations. Recently, several in vitro studies suggested synergy for polymyxin combinations including colistin combined with a carbapenem.6,7,12 However, these studies were conducted over relatively short periods (<4 days) at relatively low bacterial density (<~10^8 cfu/mL), or in one-compartment model systems that do not allow for containment of all bacterial subpopulations. However, in certain P. aeruginosa infections in critically ill patients, such as bi-lobar pneumonia, bacterial density can be remarkably high and the treatment duration is typically 10–14 days.13 Additionally, colistin activity has been shown to be attenuated at high bacterial densities.14 Therefore, it is critical to investigate the bacterial population dynamics in response to polymyxin combination regimens in an in vitro model that more closely mimics these conditions.

Here, we first characterized the temporal profile of bacterial populations for three P. aeruginosa strains in response to clinically relevant monotherapy regimens of colistin and doripenem. Studies were performed in a hollow-fibre infection model over 10 days, and in the absence of antibiotic treatment the bacterial density reached ~10^11 cfu/mL. Next, we simulated colistin and carbapenem combination regimens to study resistance suppression and characterized eight different subpopulations on colistin-containing agar plates. We developed a mathematical model based on the known mechanisms of drug action to simultaneously describe all eight bacterial subpopulations to better understand the extent, time course and potential mechanism(s) of synergy of colistin combinations. Our experimental and mathematical modelling approach provides new insights and a platform for optimizing combination regimens to combat antimicrobial resistance.

Materials and methods

Antibiotics, medium and bacterial strains

Colistin sulfate, imipenem monohydrate and ceftazidime hydrate were purchased from Sigma–Aldrich (St Louis, MO, USA) and doripenem was provided by Johnson and Johnson Pharmaceuticals. Cation-adjusted Mueller–Hinton broth (CAMHB; Difco, Detroit, MI, USA) containing calcium (25 mg/L) and magnesium (12.5 mg/L) was used. The MICs were determined by broth microdilution according to the CLSI guidelines.15 Three P. aeruginosa strains were studied: the two heteroresistant strains ATCC 27853 (colistin MIC, 1.0 mg/L; doripenem MIC, 2.0 mg/L; imipenem MIC, 2.0 mg/L; ceftazidime MIC, 2.0 mg/L) and FADDI PA033 (colistin MIC, 1.0 mg/L; doripenem MIC, 1.0 mg/L; imipenem MIC, 2.0 mg/L; ceftazidime MIC, 2.0 mg/L) and the colistin-resistant strain FADDI PA070 (colistin MIC, 128 mg/L; doripenem MIC, 2.0 mg/L; imipenem MIC, 2.0 mg/L; ceftazidime MIC, 16.0 mg/L).6,7 Colistin heteroresistance was defined as the presence of bacterial subpopulations able to grow on agar containing >2.0 mg/L colistin when the MIC was ≤2.0 mg/L.6

In vitro hollow-fibre infection model

The hollow-fibre infection model was used to simulate the time course of antibiotic concentrations observed in patients for the dosage regimens described below.16,17 Cellulosic hollow-fibre cartridges (model C3008) were purchased from FiberCell Systems, Inc. (Frederick, MD, USA). Prior to each experiment, bacterial strains were sub-cultured on cation-adjusted Mueller–Hinton agar (CAMHA; Becton, Dickinson and Company) and incubated overnight at 37°C. Bacteria from the overnight growth were added to CAMHB, and a suspension with an absorbance of 1.0 at 620 nm (equivalent to ~10^6 cfu/mL) was prepared for inoculation. Each cartridge was inoculated with ~15 mL of bacterial suspension and a pre-dose sample was collected 1 h after inoculation (time 0 h). Twenty-three samples (0.5 mL) were serially collected over 240 h. Quantification of viable counts was performed by depositing appropriately diluted bacterial samples on CAMHA plates.6,7

Simulated dosage regimens

We simulated a continuous infusion of colistin to achieve free steady-state concentrations (fC_{ss}) of 2 and 5 mg/L to mimic plasma colistin concentration–time profiles in critically ill patients.6 A colistin loading dose was given at 0 h to immediately attain the respective steady-state concentration.

To mimic the pharmacokinetics of doripenem in critically ill patients receiving 500 mg every 8 h,18 doripenem was dosed every 8 h to achieve a free maximum concentration (fC_{max}) of 25 mg/L with a simulated elimination half-life of 1.5 h.18

Population analysis profiles

Population analysis profiles were determined at pre-dose and 24, 48, 72, 96, 192 and 240 h by plating bacterial samples on CAMHA plates containing 0.5, 1, 2, 3, 4, 6, 8 or 10 mg/L colistin, as previously described.6,7,17

Impact of resistance on pathogenicity

The Galleria mellonella worm model was used to quantify the relationship between antimicrobial resistance and pathogenicity.19 The pathogenicity of each bacterial strain, prior to antibiotic exposure, was determined by injecting ~10^6 cfu (10 μL of bacterial suspension) of each strain into the left proleg; saline was utilized as a control. For each strain, a group of 10 G. mellonella weighing between 250 and 350 mg was used. The survival of G. mellonella was recorded every day for 6 days and each group was compared using Kaplan–Meier analysis in SYSTAT (version 13, Systat Software Inc., San Jose, CA, USA).

Population mathematical model

Model development

Models with multiple pre-existing subpopulations of different susceptibilities to colistin and doripenem were considered. The number of pre-existing subpopulations was selected based on model diagnostic plots, parameter values and change in objective function. The final model consisted of three subpopulations for ATCC 27853 and FADDI PA033 and two subpopulations for FADDI PA070 (Figure S1, available as Supplementary data at JAC Online). Bacterial replication was modelled using a life-cycle growth model20,21 which describes each subpopulation by a vegetative and a replicating state. The total bacterial population was modelled as the sum of all subpopulations.

A target site-binding model was used to describe polymyxin action.16 Bacterial killing was modelled as a second-order process for colistin and a first-order saturable process for doripenem (see Supplementary data available at JAC Online for details of the differential equations).

Combination activity of colistin and doripenem

Two types of synergistic interactions were considered based on the known mechanisms of action of colistin and doripenem.21–23 (i) Combinations were modelled as colistin killing the doripenem-resistant subpopulation(s) and vice versa, which was termed ‘subpopulation synergy’. This was modelled by first invoking our initial model, and subsequently refined based on modelling fits and diagnostic plots, where a number of different interactions were then incorporated. (ii) Combinations were also modelled whereby colistin enhanced doripenem’s killing and vice versa, which was termed ‘mechanistic synergy’.21 The final model included both ‘subpopulation synergy’ and ‘mechanistic synergy’.
Simultaneous modelling of the total and multiple resistant subpopulations

A new approach involving a subpopulation fraction matrix was developed to simultaneously describe the time course of viable counts on colistin-containing agar plates. The viable counts from colistin plates were described by a linear combination of the bacteria in each of the three subpopulations (i.e. CFUSS, CFUII and CFURS for strain ATCC 27853; Figure S1). Subpopulation fractions (FrColistin_conc_in_agarPOP##, such as Fr0.5PSS) were estimated to describe the fraction of bacteria of each subpopulation (#) that was quantifiable on agar plates containing a specific colistin concentration. Thus, the viable count (cfu/mL) on 0.5 mg/L colistin plates (POP0.5mg/L) was:

$$\text{POP}_{0.5\text{mg/L}} = \text{Fr}_{0.5\text{PSS}} \cdot \text{CFUSS} + \text{Fr}_{0.5\text{PIL}} \cdot \text{CFUIL} + \text{Fr}_{0.5\text{PRS}} \cdot \text{CFURS}$$

(1)

The subpopulation fractions were estimated on a log10 scale with estimates of −0 or −5, indicating that 100% or 0.001% of the respective subpopulation was viable on agar plates containing a specific colistin concentration.

The FADDI PA033 strain displayed changes in the subpopulation fractions over time. To describe phenotypic change, we incorporated a time-dependent Hill function with a maximum fold change ($E_{\text{max}}$) of the subpopulation fractions, a time associated with the half-maximal change ($T_{C0.5}$) and a Hill coefficient.

$$\Delta F_R = \frac{E_{\text{max}} \cdot \text{Time}^{Hill}}{T_{C0.5} + \text{Time}^{Hill}}$$

(2)

$$\text{Log}_{10} \left( \text{Fr}_{0.5\text{PSS}} \right) = \text{Log}_{10} \left( \text{Fr}_{0.5\text{PSS}} \right) - \Delta F_R$$

(3)

Estimation

Candidate models were estimated by simultaneously fitting the viable count profiles of all subpopulations using the importance sampling Monte Carlo parametric expectation maximization method (method = 4) in the parallelized S-ADAPT software (version 1.57) facilitated by the SADAPT-TRAN. An additive residual error model on a log10 scale was used for bacterial counts $\geq 100$ cfu/mL. To account for high sampling error at a low concentration, i.e. $< 100$ cfu/mL, an error model containing both proportion and Poisson distribution was used for the bacteria.

Additional Materials and methods for descriptive pharmacodynamics analysis, pharmacodynamics and the antibiotic activity model can be found in the Supplementary data.

Results

Rapid emergence of resistance due to colistin monotherapy

Against P. aeruginosa heteroresistant strains (ATCC 27853 and FADDI PA033), colistin monotherapy given as $fC_{ss}$ 2 mg/L demonstrated regrowth within 24 h with emergence of resistance, as shown by real-time population analysis profiles (Figures 1–3). Although increasing the exposure to colistin $fC_{ss}$ 5 mg/L resulted in initial killing up to 2.31 log10 within the first 6 h, this was followed by substantial regrowth (Figure 1 and Table S1). Although the colistin $fC_{ss}$ 2 and 5 mg/L regimens demonstrated similar killing activity against the total population, the colistin $fC_{ss}$ 5 mg/L regimen resulted in greater emergence of resistance compared with the colistin $fC_{ss}$ 2 mg/L regimen (Figures 2b and c and 3b and c).

Doripenem monotherapy given as $fC_{max}$ of 25 mg/L every 8 h achieved 1.94–4.06 log10 killing of the three strains at 6 h based on the data, but extensive regrowth was observed at 48 h for strains FADDI PA033 and FADDI PA070 (Figure 1 and Table S1). Doripenem regimens did not alter resistance emergence to colistin (Figures 2d and 3d and 4d).

Colistin combinations achieved sustained killing and resistance suppression

The combination of colistin and doripenem was markedly synergistic against all strains of P. aeruginosa. Synergy was manifested both in bacterial eradication and suppression of resistance over 240 h. Against strains ATCC 27853 and FADDI PA033, colistin at 2 mg/L plus doripenem achieved 3.69 and 4.52 log10 killing, respectively, at 6 h and maximum extents of killing of 5.08 and

Figure 1. Hollow-fibre infection model experiments with various clinically achievable dosage regimens of monotherapy and combination therapy against three strains: (a) colistin-heteroresistant ATCC 27853; (b) colistin-heteroresistant FADDI PA033; and (c) colistin-resistant FADDI PA070. Regimens simulated no antibiotic exposure (growth control), colistin given as a continuous infusion with $fC_{ss}$ of 2 or 5 mg/L, doripenem given as a bolus dose with $fC_{max}$ of 25 mg/L every 8 h with a simulated half-life of 1.5 h, 2 mg/L colistin in combination with doripenem ($fC_{max}$ of 25 mg/L dosed every 8 h) and 5 mg/L colistin in combination with doripenem ($fC_{max}$ of 25 mg/L dosed every 8 h) against an inoculum of 10 cfu/mL. Symbols represent the experimental observations and lines are the best fits based on mathematical modelling (model fit of growth control and colistin $fC_{ss}$ of 2 mg/L overlap). This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
9.02 log₁₀ relative to baseline, respectively (Figures 1a and b, 2e and 3e and Table S1). Additionally, this combination regimen suppressed the emergence of colistin resistance over 10 days and achieved 3.0–9.38 log₁₀ greater killing compared with the most active monotherapy against ATCC 27853 (Table S1 and Figure 2e). The higher colistin regimen of 5 mg/L plus doripenem completely suppressed the regrowth of resistant ATCC 27853 subpopulations over 240 h (Figure 2f), with viable counts on ≥2 mg/L colistin plates being undetectable by 144 h (Figure 2f). Against strains ATCC 27853 and FADDI PA033, this regimen led to rapid and sustained bacterial eradication (>9 log₁₀ killing within 24–48 h; Table S1 and Figures 1, 2f and 3f). Against colistin-resistant strain FADDI PA070, colistin plus doripenem combinations achieved maximum bacterial killing of 4.78 and 6.11 log₁₀ by ~72 h for the regimens containing colistin at 2 and 5 mg/L, respectively (Table S1 and Figures 1 and 4e and f). These combinations suppressed noticeable regrowth up to ~96 h.

**Pathogenicity analysis**

The survival of *G. mellonella* after bacterial challenge (Figure 5) demonstrated a greater degree of pathogenicity for reference strain ATCC 27853 compared with strains FADDI PA033 and FADDI PA070 (P<0.001 Kaplan–Meier analysis).

**Mathematical models of combination chemotherapy to characterize enhanced bacterial killing and suppression of resistance**

A mathematical model (Figure S1) based on the known mechanisms of action of colistin and bacterial physiology was successfully developed to describe the viable count profiles for all dosage regimens in monotherapy or combination therapy and all bacterial subpopulations (Figures 1–4). The coefficient of correlation for observed viable counts versus the individual (population) fitted was 0.943 (0.934), as shown in Figure S2. To mathematically model the time course of resistant bacterial subpopulations, a new approach using a subpopulation fraction matrix was developed and well characterized the bacterial signature profiles of all bacterial subpopulations over 10 days. The fraction of each bacterial subpopulation growing on colistin-containing agar plates (i.e. the subpopulation fractions; see Supplementary data) was highest for the colistin-resistant subpopulations (Figure S3).

For strain FADDI PA033, the subpopulation fractions decreased by 1.58 log₁₀ over time (Figure 3) with a time of half-maximal change of 78.4 h (Table 1). For strain ATCC 27853, the ColI subpopulation was killed rapidly by colistin. However, the ColI subpopulation was killed 43.8-fold more slowly by colistin.
monotherapy compared with the ColR subpopulation for FADDI PA033. Killing of the colistin-resistant subpopulations was very slow for all strains (Table 1). For doripenem regimens, the maximum killing rate constant of the DorI subpopulation \((K_{\text{maxI}})\) was 2.90 h^{-1} for ATCC 27853 and 3.91 h^{-1} for FADDI PA033. The doripenem concentration achieving half-maximal killing \((K_{C50})\) was ~6-fold lower for ATCC 27853 than that for FADDI PA033 (Table 1). Doripenem monotherapy was estimated to be inactive for the DorI and DorR subpopulations.

For strains ATCC 27853 and FADDI PA033, the combination was modelled as colistin effectively killing the DorI subpopulations and doripenem killing the ColR and ColI subpopulations. Colistin was also assumed to enhance doripenem’s rate of killing against the DorR subpopulation against \(P.\ aeruginosa\). Killing by colistin was not affected by the presence of doripenem. For strain FADDI PA070, the maximum killing rate constant of the DorI/DorR subpopulation by doripenem in the presence of colistin \((K_{\text{maxI/DorR}}}\) was 0.362 h^{-1} compared with 0.0 h^{-1} for \(K_{\text{maxI}}\) in monotherapy. The doripenem concentration yielding half-maximal killing \((K_{C50/DorR}}\) and \(K_{C50/ColR}\), i.e. 50% of \(K_{\text{maxI/DorR}}\) was 3.11 and 1.00 mg/L in the presence of 2 and 5 mg/L colistin, respectively. As killing of the ColR/DorR subpopulation \((K_{\text{maxR}}}\) by doripenem was unaffected by colistin, this subpopulation showed regrowth during combination therapy for strain FADDI PA070. In the presence of colistin, the maximum killing rate constant of the DorI subpopulation \((K_{\text{maxI/ColR}}}\) for doripenem was 0.203 h^{-1} for strain ATCC 27853 and 1.10 h^{-1} for strain FADDI PA033.

**Discussion**

Colistin was first introduced in the 1950s, but its use declined due to adverse effects, primarily nephrotoxicity.\(^{25,26}\) Recently, colistin has been reintroduced into clinical practice as a last-line therapy for the treatment of infections caused by Gram-negative pathogens resistant to all other antibiotics.\(^{25,26}\) However, several recent reports cite clinical failure due to the emergence of colistin resistance in \(P.\ aeruginosa\).\(^{21,22}\) Additionally, bacterial killing by colistin has been shown to be attenuated at high bacterial densities, with rapid and extensive regrowth even at colistin concentrations well above those that are clinically achievable.\(^{7,14,27,28}\) The current study shows that colistin monotherapy, even with \(f_{C_{ss}}\) of 5 mg/L (which is at the upper end of what is achievable clinically),\(^{8}\) led to rapid resistance of \(P.\ aeruginosa\). Against the ATCC 27853 strain, which was considered susceptible by MIC, colistin exposure resulted in emergence of resistant subpopulations: by 240 h, the
The entire population was resistant. Even with a lower initial inoculum of *P. aeruginosa* \(\approx 10^6\) cfu/mL, emergence of resistance during colistin monotherapy has been demonstrated with emergence of colistin-resistant subpopulations by 24–48 h. Other in vitro and animal studies corroborate our findings and show the potential for the rapid emergence of colistin resistance with monotherapy. Although strains of *P. aeruginosa* are deemed susceptible to colistin based on MICs, it is now clear that exposure to colistin monotherapy can result in rapid emergence of resistance, due to the phenomenon of colistin heteroresistance.

With regard to the development of colistin resistance due to colistin monotherapy, our temporal profiling of resistant sub-populations over 10 days suggests an enrichment of pre-existing resistant subpopulations over time. Such a phenomenon also has a higher probability of occurring at a high bacterial density since the inverse of the mutation frequency is 10-fold smaller than the initial inoculum \(\approx 10^9\) cfu/mL in the current study.

Our data also support the notion that initial dose intensity is a driver of rapidity of resistance development: greater antimicrobial intensity (colistin 5 versus 2 mg/L) resulted in faster emergence of antibiotic-resistant subpopulations. In profiling the entire population was resistant. Even with a lower initial inoculum of *P. aeruginosa* \(\approx 10^9\) cfu/mL, emergence of resistance during colistin monotherapy has been demonstrated with emergence of colistin-resistant subpopulations by 24–48 h. Other in vitro and animal studies corroborate our findings and show the potential for the rapid emergence of colistin resistance with monotherapy. Although strains of *P. aeruginosa* are deemed susceptible to colistin based on MICs, it is now clear that exposure to colistin monotherapy can result in rapid emergence of resistance, due to the phenomenon of colistin heteroresistance.

With regard to the development of colistin resistance due to colistin monotherapy, our temporal profiling of resistant sub-populations over 10 days suggests an enrichment of pre-existing resistant subpopulations over time. Such a phenomenon also has a higher probability of occurring at a high bacterial density since the inverse of the mutation frequency is 10-fold smaller than the initial inoculum \(\approx 10^9\) cfu/mL in the current study. Our data also support the notion that initial dose intensity is a driver of rapidity of resistance development: greater antimicrobial intensity (colistin 5 versus 2 mg/L) resulted in faster emergence of antibiotic-resistant subpopulations. In profiling the entire population was resistant. Even with a lower initial inoculum of *P. aeruginosa* \(\approx 10^6\) cfu/mL, emergence of resistance during colistin monotherapy has been demonstrated with emergence of colistin-resistant subpopulations by 24–48 h. Other in vitro and animal studies corroborate our findings and show the potential for the rapid emergence of colistin resistance with monotherapy. Although strains of *P. aeruginosa* are deemed susceptible to colistin based on MICs, it is now clear that exposure to colistin monotherapy can result in rapid emergence of resistance, due to the phenomenon of colistin heteroresistance.
colistin-susceptible ATCC strain, high-level resistant subpopulations (which grew on agar plates containing 10 mg/L colistin) emerged as early as 24 h when exposed to the high colistin regimen of 2 mg/L, this emergence of high-level resistance was delayed until 96 h. It has been proposed previously that *P. aeruginosa* is ‘adaptive’ under selective colistin pressure, which has been modulated by two-component regulators, including PhoP–PhoQ, PmrA–PmrB and ParR–ParS. Other mechanisms involving colistin heteroresistance may involve stochastic mutations, as in a normal bacterial replication cycle, where a replication error may occur at a frequency of $10^{-9}$ to $10^{-8}$ mutations per generation. Interestingly, in comparing the baseline virulence characteristics of the two heteroresistant strains (ATCC 27853 and FADDI PA033) in the current study, FADDI PA033 displayed a significantly increased rate of survival in the *G. mellonella* invertebrate model versus ATCC 27853. Although both strains displayed a similar population analysis profile at 0 h, the less virulent strain (FADDI PA033) displayed a greater proclivity to the emergence of resistant subpopulations under selective colistin exposure over 10 days. It is important to note that we did not examine the presence of exotoxins or the temporal relationship between emergence of resistance and alterations in pathogenicity in the studied *P. aeruginosa* isolates. However, the attenuation of virulence in heteroresistant strains has been noted in other pathogens, such as *Staphylococcus aureus*, which may suggest a potential fitness cost for development of resistance in *P. aeruginosa*.

### Table 1. Model parameter estimates

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol (unit)</th>
<th>ATCC 27853</th>
<th>FADDI PA033</th>
<th>FADDI PA070</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maximum population size</td>
<td>log$<em>{10}$ cfu$</em>{\text{max}}$</td>
<td>10.5 (1.33%)</td>
<td>10.1 (2.33%)</td>
<td>10.8 (0.848%)</td>
</tr>
<tr>
<td>Initial inoculum</td>
<td>log$<em>{10}$ cfu$</em>{0}$</td>
<td>9.11 (1.73%)</td>
<td>8.98 (2.28%)</td>
<td>9.59 (0.749%)</td>
</tr>
<tr>
<td>Log$_{10}$ (mutation frequencies)</td>
<td>Col$<em>{\text{h}}$/Dor$</em>{\text{h}}$</td>
<td>log$_{10}$ FR$_1$</td>
<td>-5.36 (4.14%)</td>
<td>-8.82 (6.81%)</td>
</tr>
<tr>
<td>Col$<em>{\text{i}}$/Dor$</em>{\text{i}}$</td>
<td>log$_{10}$ FR$_2$</td>
<td>-2.99 (9.20%)</td>
<td>-6.38 (3.85%)</td>
<td>-</td>
</tr>
<tr>
<td>Col$<em>{\text{r}}$/Dor$</em>{\text{r}}$</td>
<td>log$_{10}$ FR$_3$</td>
<td>-</td>
<td>-</td>
<td>-8.78 (2.25%)</td>
</tr>
<tr>
<td>Mean generation time</td>
<td>Col$<em>{\text{h}}$/Dor$</em>{\text{h}}$</td>
<td>MTT$_{1255}$ (min)</td>
<td>107 (10.2%)</td>
<td>155 (12.7%)</td>
</tr>
<tr>
<td>Col$<em>{\text{i}}$/Dor$</em>{\text{i}}$</td>
<td>MTT$_{1211}$ (min)</td>
<td>173 (10.2%)</td>
<td>155 (12.7%)</td>
<td>-</td>
</tr>
<tr>
<td>Col$<em>{\text{r}}$/Dor$</em>{\text{r}}$</td>
<td>MTT$_{1285}$ (min)</td>
<td>106 (10.2%)</td>
<td>155 (12.7%)</td>
<td>1120 (2.34%)</td>
</tr>
<tr>
<td>Fraction of receptors unoccupied by Ca$^{2+}$ and Mg$^{2+}$, resulting in a colistin concentration at the outer membrane target site of 50% relative to the colistin concentration in broth</td>
<td>EC$_{50}$</td>
<td>0.982</td>
<td>0.999</td>
<td>0.730</td>
</tr>
<tr>
<td>Second-order killing rate constants for colistin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>susceptible population</td>
<td>$k_2$ (L/(mg-h))</td>
<td>5.06 (12.3%)</td>
<td>6.22 (9.38%)</td>
<td>-</td>
</tr>
<tr>
<td>intermediate population</td>
<td>$k_2$ (L/(mg-h))</td>
<td>4.32 (14.2%)</td>
<td>0.194 (25.9%)</td>
<td></td>
</tr>
<tr>
<td>resistant population</td>
<td>$k_2$ (L/(mg-h))</td>
<td>0 (fixed)</td>
<td>0 (fixed)</td>
<td>0.00972 (108%)</td>
</tr>
<tr>
<td>Killing by doripenem</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>concentration needed to achieve 50% of maximum killing</td>
<td>KC$_{50}$ (mg/L)$^a$</td>
<td>0.803 (21.1%)</td>
<td>5.34 (56.5%)</td>
<td>-</td>
</tr>
<tr>
<td>with 2 mg/L colistin</td>
<td>KC$_{50CO2}$ (mg/L)</td>
<td>-</td>
<td>-</td>
<td>3.11 (16.2%)</td>
</tr>
<tr>
<td>with 5 mg/L colistin</td>
<td>KC$_{50CO5}$ (mg/L)</td>
<td>-</td>
<td>-</td>
<td>1.00 (19.7%)</td>
</tr>
<tr>
<td>maximum killing rate constants</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>for susceptible population</td>
<td>$K_{\text{maxS}}$ (h$^{-1}$)</td>
<td>2.90 (12.8%)</td>
<td>3.91 (26.7%)</td>
<td>-</td>
</tr>
<tr>
<td>for intermediate population</td>
<td>$K_{\text{maxI}}$ (h$^{-1}$)</td>
<td>0 (fixed)</td>
<td>0 (fixed)</td>
<td>0 (fixed)</td>
</tr>
<tr>
<td>for resistant population</td>
<td>$K_{\text{maxR}}$ (h$^{-1}$)</td>
<td>-</td>
<td>-</td>
<td>0.362 (9.77%)</td>
</tr>
<tr>
<td>for doripenem-intermediate population in the presence of colistin</td>
<td>$K_{\text{maxI_SYN}}$ (h$^{-1}$)</td>
<td>0.203 (31.9%)</td>
<td>1.10 (45.1%)</td>
<td>-</td>
</tr>
<tr>
<td>maximum fold change of the subpopulation coefficients</td>
<td>$E_{\text{max}}$</td>
<td>-</td>
<td>1.58 (18.9%)</td>
<td>-</td>
</tr>
<tr>
<td>time associated with the half-maximal change</td>
<td>$T_{\text{C50}}$ (h)</td>
<td>-</td>
<td>78.4 (7.45%)</td>
<td>-</td>
</tr>
<tr>
<td>standard deviation of additive error on a log$_{10}$ scale</td>
<td>SD$_{\text{cfu}}$</td>
<td>0.564 (6.53%)</td>
<td>0.577 (23.9%)</td>
<td>0.625 (6.37%)</td>
</tr>
</tbody>
</table>

$^a$KC$_{50}$ was assumed to be the same among all subpopulations for doripenem.
In summary, we characterized the detailed time course of bacterial killing and resistance of three different *P. aeruginosa* strains at a high bacterial density, in response to colistin monotherapy and combination chemotherapy over a 10 day period. An important limitation of the current work is that we studied *P. aeruginosa* organisms that were susceptible to carbapenems. Accordingly, these results should not be generalized to strains that display carbapenem resistance. Nevertheless, these findings provide an important framework for the development of a quantitative basis for optimization of colistin combination therapy and deeper insight into the time course of resistance emergence. Taken together with the proposed pharmacodynamic model based on underlying mechanisms of drug action, our approach may be useful in predicting the time course of bacterial population responses to antimicrobial combinations. The current work also has important clinical implications. As indicated by the rapid amplification of resistant subpopulations in the hollow-fibre infection model and increasing reports of clinical failure, colistin monotherapy, even at maximal doses, should not be administered in patients with infections in which there is a high bacterial density. This highlights the potential clinical utility of the studied combination of colistin and doripenem, which holds great promise for the rapid reduction of a high bacterial burden of *P. aeruginosa* when administered aggressively and early in the course of therapy. Further studies in animals employing additional strains with diverse resistance mechanisms and phenotypes are warranted to strengthen the translation of these in vitro findings into the selection of optimal dosage regimens in patients.

**Acknowledgements**

We would like to thank Pamela Kelchlin for technical support. We are grateful to Dr Arnold Louie and Dr George Drusano for insight into the hollow-fibre infection model.

**Funding**

This study was supported by the National Institutes of Health, National Institute of Allergy and Infectious Diseases (R01AI079330 and R01AI111990). J. B. B. is supported by a DECRA fellowship (DE120103084) from the Australian Research Council. N. S. L. is supported by a pre-doctoral fellowship from the American Foundation for Pharmaceutical Education. C. B. L. is supported by the Australian National Health and Medical Research Council (NHMRC, Career Development fellowship number 1062509). J. L. is an Australian National Health and Medical Research Council (NHMRC) Senior Research Fellow.

**Transparency declarations**

None to declare.

**Disclaimer**

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. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

**Supplementary data**

Supplementary data, including Table S1 and Figures S1–S3, are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

**References**


