Activity of colistin combined with doripenem at clinically relevant concentrations against multidrug-resistant Pseudomonas aeruginosa in an in vitro dynamic biofilm model

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Objectives: Colistin combination therapy may be required to treat biofilm-associated infections. We evaluated bacterial killing and emergence of colistin resistance with colistin and doripenem combinations against biofilm-embedded and planktonic multidrug-resistant (MDR) Pseudomonas aeruginosa.

Methods: One colistin-susceptible reference strain (PAO1) and two colistin-susceptible MDR clinical isolates (HUB1 and HUB2; both carbapenem resistant) were investigated over 72 h in the CDC biofilm reactor, a dynamic biofilm model. Two colistin regimens (constant concentrations of 1.25 and 3.50 mg/L), one doripenem regimen ($C_{\text{max}}$ 25 mg/L 8 hourly) and their combination were employed. Microbiological response was examined as log changes and absolute bacterial counts.

Results: For biofilm-embedded bacteria, bactericidal activity was only observed with monotherapy with colistin at 3.50 mg/L. The emergence of colistin resistance occurred with colistin monotherapy against two strains (PAO1 and HUB1), but only with the colistin 3.50 mg/L regimen. Colistin 3.50 mg/L plus doripenem resulted in $\sim$2–3 log_{10} cfu/cm² initial killing against both clinical isolates and remained synergistic at 72 h. The emergence of colistin resistance was not observed in biofilm-embedded bacteria with either combination. For planktonic bacteria, bactericidal activity was not observed with any monotherapy regimen, although enhanced bacterial killing was observed with doripenem plus colistin 3.50 mg/L against all isolates. Colistin resistance was observed with colistin monotherapy against two isolates, but did not emerge with combination regimens.

Conclusions: Doripenem enhanced killing by colistin of biofilm-embedded cells in both carbapenem-susceptible and -resistant strains, and the combination minimized the emergence of colistin resistance.

Keywords: foreign body, polymyxins, carbapenem resistant, CDC reactors, PK/PD, infections

Introduction

Rapidly increasing antibiotic resistance and a dearth of new antibiotics in the drug development pipeline represent a major global medical challenge.1,2 Infections by multidrug-resistant (MDR) Gram-negative bacilli such as Pseudomonas aeruginosa are particularly problematic, with no new antibiotics to treat these infections to become available in the foreseeable future.3,4 MDR P. aeruginosa has been identified by the Infectious Diseases Society of America (IDSA) as one of the top six pathogens threatening healthcare systems.1,4 As a versatile pathogen with the ability to cause diverse types of infection, P. aeruginosa is of central importance in a broad range of nosocomial and community-acquired infections, including biofilm-associated infections.5–9 ‘Old’ polymyxins, particularly colistin, are often the only therapeutic option.4,10–13

Colistin is administered parenterally in the form of sodium colistin methanesulphonate (CMS), an inactive prodrug.14 However, the emerging pharmacokinetic (PK) and pharmacodynamic (PD) data suggest that caution is required with the use of colistin monotherapy due to suboptimal exposure and the emergence of resistance.15–17 Both in vitro18–25 and in vivo26–28 studies have shown the potential for the rapid emergence of colistin resistance with monotherapy, including against P. aeruginosa,
very likely due to amplification of pre-existing colistin-resistant subpopulations.\textsuperscript{19,21,22,29} Such observations highlight the importance of investigating rational combinations of colistin with other antibiotics to minimize the emergence of colistin resistance.\textsuperscript{19,21}

Treatment of biofilm-related infections caused by MDR \textit{P. aeruginosa}, including those associated with a foreign body, is particularly problematic and the clinical prognosis is poor.\textsuperscript{7,30,31} Biofilms are complex bacterial communities embedded in a self-produced polymeric matrix that protects the cells from environmental, immune system and antimicrobial threats.\textsuperscript{32,33} Bacterial cells growing in a biofilm may become substantially more resistant to antibiotic treatment than planktonic cells\textsuperscript{34,35} and a phenomenon contributing to the chronicity of MDR bacterial infections.\textsuperscript{33,36} Although significant synergy has been reported for the combination of polymyxin and a carbapenem against non-biofilm infections caused by \textit{P. aeruginosa},\textsuperscript{19,21,37} for biofilm-related infections there is a paucity of PK/PD information regarding such combinations.\textsuperscript{38–43} Thus, the aim of the present study was to investigate the activity of colistin alone and in combination with doripenem with respect to bacterial killing and the emergence of resistance in biofilm-embedded carbapenem-resistant \textit{P. aeruginosa} by simulating the PK of both antibiotics in humans using an in vitro dynamic biofilm model.

### Materials and methods

#### Bacterial isolates

Three colistin-susceptible but heteroresistant strains of \textit{P. aeruginosa} were employed in this study: the carbapenem-susceptible reference strain PAO1 (American Type Culture Collection, Rockville, MD, USA) and two clonally unrelated carbapenem-resistant clinical isolates: HUB1 (extensively drug-resistant, XDR) and HUB2 (MDR). Heteroresistance to colistin was defined as having an MIC ≤ 2 mg/L and with subpopulations able to grow in the presence of ≥ 4 mg/L colistin in population analysis profiles (PAPs; determined as described previously).\textsuperscript{19,21} XDR was defined as having an MIC ≥ 4 mg/L colistin in population analysis profiles (PAPs; determined as described previously).\textsuperscript{19,21} XDR was defined as having an MIC ≥ 4 mg/L colistin in population analysis profiles (PAPs; determined as described previously).\textsuperscript{19,21} Both clinical isolates caused outbreaks in the Hospital Universitario de Bellvitge in Barcelona, Spain, and contained a VIM-2 metallo-β-lactamase (HUB1)\textsuperscript{35} or a PSE-1 β-lactamase plus a MexXY-OprM efflux pump (HUB2).\textsuperscript{46} MICs of colistin (sulphate) and doripenem for each isolate are shown in Table 1 and were determined using broth microdilution in cation-adjusted Mueller–Hinton broth (CAMHB) and cation-adjusted 1% Tryptone soy broth (CA-1%TSB) for both media, Ca\textsuperscript{2+} at 23.0 mg/L and Mg\textsuperscript{2+} at 12.2 mg/L; Oxoid, Basingstoke, UK).\textsuperscript{57} Determination of MICs in CAMHB was performed for comparison with those observed in CA-1%TSB, which was the growth medium used in our model. Resistance to colistin\textsuperscript{37} and doripenem\textsuperscript{48} in \textit{P. aeruginosa} was defined as MIC ≥ 4 mg/L. Isolates were stored in tryptone soy broth (Oxoid) with 20% glycerol (Ajax Finechem, Seven Hills, New South Wales, Australia) in cryovials (Simport Plastics, Quebec, Canada) at −80°C.

#### Antibiotics and reagents

For MIC determinations and in vitro PK/PD studies, colistin sulphate (C4461, lot number SLBD8306V; ≥ 15 000 U/mg) and doripenem (lot 0137Y01) were used (Sigma-Aldrich, Castle Hill, Australia). Colistin sulphate was employed in the current study as colistin is the active antibacterial entity formed in vivo after administration of its inactive prodrug, CMS.\textsuperscript{34} Stock solutions of colistin and doripenem were prepared immediately prior to each experiment using Milli-Q water (Millipore Australia) and 0.9% saline, respectively, and sterilized by filtration with a 0.20 μm cellulose acetate syringe filter (Millipore, Bedford, MA, USA).

### Binding of colistin and doripenem in CA-1%TSB

The binding of colistin and doripenem in CA-1%TSB was determined by ultracentrifugation (Optimal MAX-TL, Beckman Coulter, Inc.). Colistin and doripenem were spiked in CA-1%TSB at 3.5 and 25 mg/L, respectively. An aliquot (200 μL) of drug-containing CA-1%TSB was transferred to centrifuge tubes (poly carbonate, 7 × 20 mm, Beckman Coulter, Inc.) and incubated for 30 min at 37°C (n = 3). Tubes were then subjected to ultracentrifugation using a TLA-100 fixed-angle rotor (Beckman Coulter, Inc.) at 279 000 g for 4 h at 37°C, and 50 μL was removed from the upper part of the supernatant of two replicate tubes. The contents of the third tube were resuspended and two 50 μL samples were removed from the central part of the tube. All samples were stored at −80°C until analysis. Concentrations of colistin and doripenem were determined in two replicates by HPLC.\textsuperscript{21,49} The percentage of colistin and doripenem bound in CA-1%TSB was calculated as follows:

\[
\text{Binding percentage} = \left(1 - \frac{\text{Supernatant concentration}}{\text{Resuspended concentration}}\right) \times 100%
\]

The final binding percentage was calculated as the average of two values.

### In vitro PK/PD biofilm model

Experiments to examine bacterial killing and the emergence of resistance to various dosage regimens of colistin and doripenem alone and in combination were conducted over 72 h using a CDC biofilm reactor (CBR) (BioSurface Technologies, Bazemans, MT, USA). This dynamic model consisted of a 1 L glass reactor connected to a 10 L carboy containing sterile drug-free CA-1%TSB. The broth was pumped through the model with mixing and shear generated by a magnetic stir bar operating at 130 rpm. The volume of broth in the glass reactor was maintained at 350 mL and a waste vessel was connected. Eight polypropylene coupon holders were suspended from the lid, each containing three removable Teflon coupons (diameter 12.7 mm) on which biofilm formed. The biofilm-growing surface on the two faces of each coupon totalled 2.53 cm\textsuperscript{2}. Our protocol for biofilm growth was based upon previously published methods.\textsuperscript{50,52} Briefly, prior to each experiment isolates were subcultured onto nutrient agar plates and incubated at 35°C for 24 h. One colony was then selected and grown overnight in 10 mL of TSB, from which early log-phase growth was obtained. A 1 mL aliquot of this early log-phase bacterial suspension was inoculated into the model at 37°C and a 28 h conditioning phase commenced. This consisted of 24 h of incubation in drug-free CA-1%TSB, after which the model was emptied and fresh sterile drug-free CA-1%TSB was employed in the current study as colistin is the active antibacterial entity formed in vivo after administration of its inactive prodrug, CMS.\textsuperscript{34} Stock solutions of colistin and doripenem were prepared immediately prior to the next experiment using Milli-Q water (Millipore Australia) and 0.9% saline, respectively, and sterilized by filtration with a 0.20 μm cellulose acetate syringe filter (Millipore, Bedford, MA, USA).

### Table 1. MICs (mg/L)\textsuperscript{a} for the \textit{P. aeruginosa} isolates examined in this study

<table>
<thead>
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<th>Colistin</th>
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<th>Doripenem</th>
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<tr>
<td></td>
<td>CAMHB</td>
<td>CA-1%TSB</td>
<td>CAMHB</td>
</tr>
<tr>
<td>PAO1</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>HUB1</td>
<td>2</td>
<td>2</td>
<td>&gt;128</td>
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<tr>
<td>HUB2</td>
<td>1</td>
<td>2</td>
<td>16</td>
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\textsuperscript{a}CLSI breakpoints for colistin were ≤2 mg/L for susceptibility, 4 mg/L for intermediate and ≥8 mg/L for resistance. For doripenem, the breakpoints were ≤2 mg/L for susceptibility and >2 mg/L for resistance.\textsuperscript{47}
pumped into the model at a flow rate of 11.67 mL/min for 4 h prior to the commencement of antibiotic treatment (i.e. 0 h). For viable counting and examination of emergence of colistin resistance in the biofilm-embedded cells, three coupons were removed aseptically at each of 0, 4, 8, 24, 32, 48, 56 and 72 h, rinsed twice in 5 mL of phosphate-buffered saline (PBS, pH 7.4) to remove planktonic cells, and placed in sterile tubes containing 10 mL of PBS. Biofilm-embedded cells were recovered by three alternating 1 min cycles of vortexing and sonication at 43 kHz (Soniclean, Therbaton, Australia) followed by a final 1 min vortexing.52 CA-1%TSB (1 mL) was also removed from the model at each timepoint for counting of viable cells and examination of emergence of colistin resistance in planktonic cells (below).

Two colistin regimens and one doripenem regimen were examined as monotherapy and as their respective combinations. Colistin was simulated as a continuous infusion at 1.25 or 3.50 mg/L. This was achieved by bolus administration of colistin at 0 h to the model to achieve the desired concentration of 1.25 or 3.50 mg/L, as well as by spiking the medium in the carboy with colistin at the appropriate concentration. Our approach mimicked the flat plasma concentration–time profiles of formed colistin at steady state observed in critically ill patients receiving CMS.15–17,51 For doripenem regimens, a bolus dose was introduced into the model every 8 h to achieve the desired steady-state peak concentration ($C_{max}$) of 25 mg/L. The flow rate to the glass reactor vessel (4 mL/min) was chosen to simulate a doripenem elimination half-life ($t_{1/2}$) of 1 h in patients.56 Dosage regimens of both colistin and doripenem represent clinically achievable unbound (free) plasma concentration–time profiles in patients. All control and drug-containing regimens except those containing colistin at 3.50 mg/L (as monotherapy or in combination) were performed in two replicates, with three coupons examined at each timepoint per run (i.e. six coupons in total); additionally, two broth samples from each model were collected at each timepoint for enumeration of planktonic cells (below). For regimens involving colistin at 3.50 mg/L, one experiment was conducted with three coupons per timepoint for measurements of biofilm-embedded cells and one sample for assessing planktonic cells. Flow rates were calibrated prior to each experiment and monitored throughout the experiment to ensure the system was performing optimally.

Microbiological response and emergence of resistance to colistin

For enumeration of biofilm-embedded and planktonic viable cells, the respective samples were serially diluted with sterile saline and 50 µL was spirally plated onto drug-free nutrient agar (Media Preparation Unit) using an automatic spiral plater (WASP, Don Whitley Scientific, West Yorkshire, UK). Serial 10-fold dilutions and spiral plating, which further diluted the samples, minimized antibiotic carryover. Colonies were counted using a ProtoCOL automated colony counter (Synbiosis, Cambridge, UK) after 24 h of incubation at 35 °C and 48 h for the plates with small colonies. In order to evaluate the emergence of colistin resistance, both biofilm-embedded and planktonic (broth) samples were additionally plated in a similar manner onto nutrient agar containing 4 mg/L colistin (Media Preparation Unit).

PK validation

Samples (100 µL) collected in duplicate from the model were placed in 1.5 mL microcentrifuge tubes (Greiner Bio-One, Frickenhausen, Germany) and immediately stored at −80 °C; all samples were assayed within 4 weeks to avoid any potential degradation. Concentrations of colistin and doripenem were measured using HPLC as previously described,21,49 with assay ranges of 0.10–6.00 mg/L for colistin and 0.5–32 mg/L for doripenem. For both colistin and doripenem assays, analysis of quality control samples revealed that measured and nominal concentrations differed by <±10% and coefficients of variation were <10.2%.

PD analysis

Microbiological responses of mono- or combination regimens were examined using the log change method, i.e. comparing the change in log_{10} cfu/cm² (biofilm-embedded cells) or log_{10} cfu/mL (planktonic cells) from 0 h ($C_{f0}$) to time $t$ (4, 8, 24, 32, 48, 56 or 72 h; $C_{fu}$) as shown: log change = log$_{10}(C_{fu})$ − log$_{10}(C_{f0})$.19,21 Treatments were considered to be bactericidal (99.9% kill) when they led to a ≥3 log$_{10}$ cfu/cm² or cfu/mL reduction, compared with the corresponding counts at zero time. Monotherapy or combination regimens causing a reduction of ≥1 log$_{10}$ cfu/cm² or cfu/mL at a specified time were considered active. Synergy was defined as ≥2 log$_{10}$ cfu/cm² or cfu/mL killing for the combination relative to the most active corresponding monotherapy at a specified time,55 additivity was defined as 1 to <2 log$_{10}$ cfu/cm² or cfu/mL greater killing for the combination. The emergence of colistin resistance (i.e. ability to grow on plates with a colistin concentration of 4 mg/L) was examined using absolute bacterial counts (log$_{10}$ cfu/cm² or log$_{10}$ cfu/mL) for biofilm-embedded and planktonic cells, respectively.

Results

PK validation and binding of colistin and doripenem in CA-1%TSB

The achieved colistin concentrations (mean ± SD) were 1.20 ± 0.18 (n = 55) and 3.80 ± 1.03 (n = 30) mg/L for the targeted concentrations of 1.25 and 3.50 mg/L, respectively. Measured doripenem $C_{max}$ values were 20.3 ± 3.08 (n = 90) for the targeted value of 25.0 mg/L. The observed mean $t_{1/2}$ for the simulated intermittent doripenem dosage regimen was 1.02 ± 0.11 h (n = 15) for the targeted value of 1.0 h. The percentages of colistin and doripenem bound in CA-1%TSB (2.00% and 0%, respectively) were negligible, indicating practical equivalence of total and bound concentrations. The percentage of time that unconbound concentrations of doripenem exceeded the MIC ($f_{T>MIC}$) was 100%, 0% and 21% for PAO1, HUB1 and HUB2, respectively. The area under the unconbound colistin concentration–time curves over 24 h divided by the MIC ($f_{AUC/MIC}$) for the three strains was 14.4 and 45.6 for the regimens of colistin 1.25 and 3.50 mg/L, respectively.

Microbiological response

The baseline PAPs of all isolates are presented in Figure 1. The presence of colistin-resistant subpopulations is evident prior to colistin treatment. The time-course profiles of bacterial numbers of biofilm-embedded and planktonic bacteria for control (drug-free) experiments are shown in Figure 2. Log changes in viable cell counts in the presence of colistin, doripenem or the combination, and the emergence of colistin-resistant bacteria are shown in Figure 3 (biofilm-embedded cells) and Figure 4 (planktonic bacteria).

The colistin monotherapy regimen at 1.25 mg/L was ineffective against biofilm-embedded PAO1 bacteria and produced only modest, non-bactericidal killing of HUB1 and HUB2 (Figure 3). The high-concentration colistin monotherapy regimen (3.50 mg/L) produced greater and more rapid initial killing of biofilm-embedded bacteria of all strains, but with subsequent regrowth by 72 h such that bactericidal activity was only observed at this time against HUB1. The 3.50 mg/L colistin monotherapy regimen against PAO1 and HUB1 resulted in the emergence of colistin resistance within the biofilm; however, no colistin-resistant colonies of HUB2 were
detected with either colistin regimen. For all isolates the emergence of colistin resistance was more pronounced in planktonic bacteria (Figure 4, bottom panels). Doripenem monotherapy achieved rapid and sustained killing (although not bactericidal) against biofilm-embedded PAO1 across 72 h (Figure 3, top panels), but against planktonic bacteria regrowth occurred rapidly following initial killing (Figure 4, top panels). Not surprisingly, doripenem monotherapy was ineffective against the carbapenem-resistant isolates (HUB1 and HUB2).

The combination of colistin 1.25 mg/L plus doripenem showed some additive effects against biofilm-embedded bacteria during the first 24–32 h of treatment, especially against PAO1 (Figure 3, top panels). However, this combination was not bactericidal in biofilm against any strain and against both clinical isolates it was generally no better than the most active colistin monotherapy. Against planktonic PAO1, additivity was observed across 72 h with this combination; however, against the clinical isolates the only benefit over colistin monotherapy was additivity within the first 24 h against HUB2 (Figure 4, top panels). The emergence of colistin-resistant subpopulations was only observed with HUB2 at 48 h in planktonic bacteria (Figure 4, bottom panels).

The combination of colistin 1.25 mg/L plus doripenem resulted in greater and more sustained killing than either corresponding monotherapy across 72 h. Against biofilm-embedded PAO1 this combination was synergistic up to 48 h and additive at 56 and 72 h, while against both clinical isolates greater initial killing (of \( \approx 2-3 \log_{10} \text{cfu/cm}^2 \) compared with equivalent monotherapy) was followed by slow regrowth, but nevertheless remained synergistic at 72 h (Figure 3, top panels). Against planktonic bacteria this combination produced \( \approx 1.5 \log_{10} \text{cfu/mL} \) greater bacterial killing than with the corresponding monotherapy for all strains, with primarily synergistic (PAO1 and HUB2) or additive (HUB1) effects observed across the 72 h (Figure 4, top panels). No emergence of colistin resistance was observed with this combination in either biofilm-embedded or planktonic bacteria (Figures 3 and 4, bottom panels).

Figure 1. Baseline PAPs of the reference strain PAO1 and clinical isolates HUB1 and HUB2 at an initial inoculum of \( \approx 10^9 \text{cfu/mL} \). The y-axis starts from the limit of quantification. The log10 cfu/mL values for PAO1 and HUB1 at \( \geq 4 \text{mg/L} \) are below the limit of quantification.

Figure 2. Bacterial growth in the absence of colistin and doripenem (i.e. growth controls) for biofilm-embedded (a) and planktonic (b) bacteria for the three strains of *P. aeruginosa*. Time on the x-axis begins immediately after the 28 h conditioning phase. The y-axis starts from the limit of quantification. Data are presented as means ± SD (a) or as means (b).

**Discussion**

Foreign-body infections by MDR *P. aeruginosa* are of great clinical concern, with a limited number of therapeutic options currently available. Increasingly, colistin is being used as a last-line therapy for treatment of such infections.\(^4,7,11,50,46,56\) However, the emergence of colistin resistance has been reported in *P. aeruginosa in vitro* with colistin monotherapy;\(^19–21,57–59\) regrowth is due, at least in part, to amplification of pre-existing colistin-resistant subpopulations.\(^19,20\) Additionally, recent studies indicate that the plasma colistin concentrations achieved in critically ill patients with the currently recommended CMS dosage regimens are suboptimal in many cases.\(^15,17\) Following intravenous administration of CMS, total (i.e. bound and unbound) plasma colistin concentrations of \( \approx 2-3 \text{mg/L} \) are typically achieved at steady state, with some patients achieving concentrations of up to \( \approx 10 \text{mg/L}.\)\(^15–17,53\) We administered colistin as a continuous infusion to simulate the flat profiles of formed colistin observed in critically ill patients at steady
state across a CMS dosage interval.\textsuperscript{15,17} Similarly, doripenem is typically administered 8 hourly with peak concentrations of \( \approx 25 \) mg/L achieved after a standard 500 mg dose.\textsuperscript{48} Binding of doripenem to plasma proteins is very low (\( \approx 8\%)\textsuperscript{48} while the bound fraction of colistin in human plasma is \( \approx 50\%).\textsuperscript{16} Given the minimal binding of both colistin and doripenem in CA-1\%TSB, the dosage regimens of colistin and doripenem employed in the present study reflect clinically achievable unbound (free) plasma concentration–time profiles in patients (the 1.25 and 3.50 mg/L colistin concentrations used in these experiments would be equivalent to total concentrations of \( \approx 2.50 \) and 7 mg/L, respectively).

The difficulties of achieving adequate colistin concentrations, as outlined above, are exacerbated with biofilm infections, in which MICs and minimum bactericidal concentrations (MBCs) are substantially increased.\textsuperscript{39,40} Previous in vitro\textsuperscript{38,60,61} and in vivo\textsuperscript{39} studies have demonstrated the need for very high concentrations of colistin when used as monotherapy to achieve any substantial killing of biofilm-embedded bacterial cells. In a recently published study utilizing a mouse lung infection biofilm model, a colistin serum concentration of \( 64 \times \text{MIC} \) (i.e. 128 mg/L) was required to achieve a 1 log\(_{10}\) decrease in cfu/lung.\textsuperscript{39} This concentration far exceeds the upper limits of clinically achievable colistin concentrations (~10 mg/L) following intravenous administration of CMS.\textsuperscript{15,17,60} and alternative strategies must therefore be applied in order to adequately treat biofilm infections caused by MDR \textit{P. aeruginosa}.

Combination therapy has been suggested as a promising approach to increase bacterial killing and minimize the emergence of colistin resistance.\textsuperscript{15,19,21,38,61} Previous studies have investigated the PD of colistin/carbapenem combinations in planktonically grown isolates of colistin-susceptible and -resistant \textit{P. aeruginosa} (including carbapenem-resistant strains) using static and dynamic time–kill methods.\textsuperscript{19,21,39} These studies demonstrated increased bacterial killing and a reduction or prevention of the emergence of colistin resistance with this combination, the activity being greater with imipenem and doripenem than with meropenem against \textit{P. aeruginosa}.\textsuperscript{37} In combination, the activity of colistin and a second antibiotic may be complementary by targeting distinct bacterial subpopulations with different antimicrobial susceptibilities. In addition to this so-called subpopulation synergy (where different drugs target cells with different susceptibilities), mechanistic synergy has also been proposed for combinations involving colistin, whereby each drug acts on different metabolic pathways or otherwise enhances killing by the second drug.\textsuperscript{62} For the latter situation, it has been suggested that the ability of colistin to disrupt the Gram-negative outer membrane, increasing its permeability, may allow greater access of doripenem to the critical penicillin-binding proteins located on the cytoplasmic membrane, where the carbapenems act.\textsuperscript{21}

Bacterial cells growing in a biofilm differ from planktonic cells in a number of important ways and metabolically distinct

Figure 3. (Top panels) Bacterial killing by colistin (CST) alone at two different clinically relevant concentrations and by doripenem (DOR) alone and in combination against biofilm-embedded cells of three different \textit{P. aeruginosa} strains. Results are expressed using the log change method. (Bottom panels) Emergence of colistin resistance (i.e. colonies able to grow in the presence of \( \geq 4 \) mg/L colistin) among biofilm-embedded \textit{P. aeruginosa} across the treatment period with the same treatment regimens. Results are expressed as the absolute number of recovered bacteria. In the bottom panels, the y-axis starts from the limit of quantification. Data are presented as means \( \pm \) SD.

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\caption{(Top panels) Bacterial killing by colistin (CST) alone at two different clinically relevant concentrations and by doripenem (DOR) alone and in combination against biofilm-embedded cells of three different \textit{P. aeruginosa} strains. Results are expressed using the log change method. (Bottom panels) Emergence of colistin resistance (i.e. colonies able to grow in the presence of \( \geq 4 \) mg/L colistin) among biofilm-embedded \textit{P. aeruginosa} across the treatment period with the same treatment regimens. Results are expressed as the absolute number of recovered bacteria. In the bottom panels, the y-axis starts from the limit of quantification. Data are presented as means \( \pm \) SD.
\end{figure}
subpopulations within the biofilm structure have been identified with varying susceptibilities to specific antimicrobials. For example, bacterial cells with lower metabolic activity located in deeper layers of the biofilm may retain susceptibility to colistin, but not to other antibiotics. Indeed, the combination of colistin with tobramycin has shown benefits in an in vitro biofilm model. To the best of our knowledge, our study is the first to investigate the activity of colistin/carbapenem combinations against biofilm-embedded P. aeruginosa using an in vitro dynamic biofilm model. The CDC biofilm reactor is a well-accepted, validated tool for performing in vitro PK/PD experiments involving biofilm, allowing simulation of clinically relevant PK. In our model we utilized 1%TSB, a nutrient-restricted medium with which the growing of P. aeruginosa biofilm has been standardized in the CDC biofilm reactor. Furthermore, the cation concentrations in 1%TSB were adjusted, as concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) can affect colistin antibacterial activity.

In agreement with previous non-biofilm studies involving colistin regimens, regrowth was generally observed with colistin monotherapy against biofilm-embedded cells (Figure 3, top panels). The presence of colistin heteroresistance in all isolates (Figure 1) may explain these observations, at least in part, as amplification of pre-existing colistin-resistant subpopulations likely contributed to regrowth. This further supports the view that colistin should not be used as monotherapy against P. aeruginosa, especially in the setting of foreign-body infections. The diminished activity of β-lactams in the setting of foreign-body and biofilm infections has been reported previously, and was similarly observed here against the doripenem-susceptible reference isolate. As expected, no bacterial killing was observed for the two clinical isolates with doripenem monotherapy. However, against biofilm-embedded cells the addition of doripenem to colistin resulted in synergy in bacterial killing over corresponding monotherapy against all three isolates across the 72 h, primarily with the 3.50 mg/L colistin combination regimen. Similar to the results achieved against biofilm-embedded bacteria, improvements in bacterial killing of planktonic bacteria were primarily observed with the combination regimen containing 3.50 mg/L colistin. As in our previous investigation with colistin/doripenem combinations against planktonic P. aeruginosa, this combination eliminated or reduced the emergence of colistin resistance.

There are a number of possible reasons for the strain-to-strain differences in both biofilm-embedded and planktonic bacterial killing observed in this investigation (Figures 3 and 4). Firstly, biofilm-forming ability and particular biofilm characteristics are strain-dependent. The three strains used in this investigation established biofilms with varying initial cell densities following the conditioning phase (Figure 2). Second, the different PAPs observed for the three strains prior to antibiotic treatment (Figure 1) indicate slightly different frequencies of pre-existing colistin-resistant
subpopulations at the commencement of therapy. Finally, the two clinical isolates had a different mechanism(s) of carbapenem resistance (a carbapenemase in HUB1 and an efflux pump plus a β-lactamase in HUB2). The interplay of these factors likely contributed to the strain differences in the bacterial killing observed.

The use of our in vitro PK/PD biofilm model deserves some additional comments. First, the killing of planktonic cells by the mono- and combination therapy in the present study was reduced in comparison with that observed in our previous PK/PD study with comparable colistin and doripenem exposures. It is very likely that the reduced efficacy was due to continuous release of bacterial cells from the biofilm in the CDC model. In addition, the antimicrobial treatment in our experiments commenced after 28 h of biofilm growth. This must be taken into account when interpreting the results, since the maturity of the biofilm has an important influence on the activity of antimicrobials.

In summary, we have shown for the first time that clinically relevant dosage regimens of colistin and doripenem in combination increase bacterial killing of biofilm-embedded P. aeruginosa, including carbapenem-resistant isolates, with negligible emergence of colistin resistance. These findings provide important PK/PD information for foreign-body infections caused by MDR P. aeruginosa. Further investigations using validated animal biofilm models are warranted and are currently under way in our laboratory.

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Transparency declarations
None to declare.

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