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Towards optimising use of colistin against *Pseudomonas aeruginosa*

A thesis submitted for the degree of
DOCTOR OF PHILOSOPHY

by

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Drug Delivery, Disposition and Dynamics
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*This thesis is dedicated to my beautiful wife Sarah,
my adorable children Timothy and James,
and first and foremost to my LORD and saviour Jesus Christ.*

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Abstract

The use of colistin, a 50-year old polymyxin antibiotic with a reportedly high incidence of nephrotoxicity and neurotoxicity, declined with the development of potentially less toxic anti-pseudomonal agents. Colistin retains significant activity against emerging multidrug-resistant (MDR) Gram-negative bacteria and is often the only therapeutic option available to treat infections by these pathogens. As a consequence, use of colistin as a 'last-line' of defence has increased dramatically over recent years. However, there has been a dearth of pharmacological information to inform rational use of colistin in order to maximise antibacterial activity and minimise toxicity and the development of resistance. As resistance to colistin is beginning to emerge, there is an urgent need to optimise use of colistin to prolong its therapeutic utility. To increase our understanding of colistin pharmacokinetics (PK) and pharmacodynamics (PD) this thesis examined the activity of colistin against *Pseudomonas aeruginosa*.

Colistin is administered parenterally as its sulphomethylated derivative, sodium colistin methanesulphonate (CMS); colistin is subsequently formed from CMS *in vivo*. The contribution to bacterial killing of both CMS and formed colistin was unknown, despite the important implications for susceptibility and PK/PD studies. The relative antibacterial activity of CMS and colistin against *P. aeruginosa* was thus investigated. The time-course of the killing effect achieved with CMS (and formed colistin) was very similar to that observed with colistin when added to achieve the same colistin concentration-time course resulting from the conversion of CMS. Killing with CMS did not begin until significant concentrations of colistin were achieved, indicating that CMS possesses little antibacterial activity. As the time-course of antibacterial activity from CMS could be accounted for by the appearance of colistin, it was clearly

demonstrated that antipseudomonal activity was due to formation of colistin. CMS may therefore be regarded as an inactive pro-drug of colistin.

Confusion surrounds the optimal dosing regimen of CMS. Three clinically relevant dosage regimens of CMS (8, 12 and 24 hourly) were simulated in an *in vitro* PK/PD model to evaluate the PD of colistin against *P. aeruginosa*. Overall bacterial killing and regrowth throughout the experimental period (72 h) was generally similar among the three regimens, with regrowth observed with all regimens. Population analysis profiles (PAPs) revealed the presence of colistin-resistant subpopulations with each regimen at 72 h. In the two regimens which employed the greater dosage interval (12 and 24 h), the emergence of resistance was substantially greater and occurred earlier than for the 8 hourly regimen.

Information on the PK/PD index that best predicts colistin efficacy is important for rational design of optimal dosing strategies. Dose fractionation was employed in an *in vitro* PK/PD model to identify the PK/PD index (i.e., the area under the unbound concentration-time curve to MIC ratio [$fAUC/MIC$], the unbound maximal concentration to MIC ratio [fC_{max}/MIC], or the cumulative percentage of a 24-h period that unbound concentrations exceed the MIC [$fT_{>MIC}$]) that best predicts colistin efficacy against *P. aeruginosa* and to determine the values for the predictive index required to achieve various magnitudes of killing effect. Overall killing effect was best correlated with $fAUC/MIC$. The magnitudes of $fAUC/MIC$ required for 1- and 2- \log_{10} reductions in the area under the cfu/mL curve relative to growth control were identified.

Use of combination antibiotic therapy may be beneficial against rapidly emerging resistance in *P. aeruginosa*. Bacterial killing and resistance emergence with colistin monotherapy and in combination with imipenem was systematically investigated at two inocula ($\sim 10^6$ and $\sim 10^8$ cfu/mL) using static time-kill methodology. The bacterial strains examined included colistin-heteroresistant and colistin- and imipenem-resistant strains; colistin-heteroresistant

P. aeruginosa was first identified in this study. Colistin combined with imipenem at clinically achievable concentrations substantially increased bacterial killing against MDR and colistin-heteroresistant isolates at both inocula. Combination therapy against colistin-susceptible isolates generally had little effect on the proportion of colistin-resistant subpopulations, with PAPs very similar to that obtained with equivalent colistin monotherapy.

Finally, to further investigate the combination of colistin and carbapenems, a systematic investigation examining the bacterial killing and emergence of colistin resistance with colistin alone and in combination with doripenem at both high and low inocula against *P. aeruginosa* was undertaken using an *in vitro* PK/PD model. The addition of doripenem to colistin using clinically relevant dosage regimens resulted in substantial improvements in bacterial killing over equivalent monotherapy against the MDR colistin-resistant isolate at both inocula. Although 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. Combination therapy substantially reduced and delayed the emergence of colistin-resistant subpopulations in the colistin-susceptible strain, but had no effect on the colistin resistance of a MDR colistin-resistant isolate.

In brief, this thesis was the first to demonstrate CMS is an inactive prodrug of colistin and to systematically investigate colistin combinations. The findings contained therein improve the current understanding of the PK/PD determinants of colistin activity and resistance development. This work provides important information that will assist in designing optimal dosing strategies to maximise the efficacy of, and reduce the development of resistance to, this increasingly important therapeutic agent.

Statement of originality

In accordance with Doctorate Regulation 17 of Monash University, the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes five original papers (Chapters 2 to 6) published or accepted in peer reviewed journals. The design of the studies, laboratory experiments, data analysis and interpretation, development and writing of the papers included in this thesis, and formulation of conclusions and hypotheses arising from the results of the studies, were the principle responsibility of myself, the candidate, working within the Facility for Anti-infective Drug Development and Innovation, under the supervision of Prof. Roger L. Nation and Assoc Prof. Jian Li.

The inclusion of co-authors reflects the fact that the work was derived from active collaborations between researchers, and acknowledges input into team-based research.

Thesis chapter	Publication title	Publication status	Extent of candidate's contribution
2	Colistin methanesulphonate is an inactive prodrug of colistin against <i>Pseudomonas aeruginosa</i>	Published	65%
3	Comparison of once-, twice- and thrice-daily dosing of colistin on antibacterial effect and emergence of resistance: studies with <i>Pseudomonas aeruginosa</i> in an <i>in vitro</i> pharmacodynamic model	Published	70%
4	Pharmacokinetic/Pharmacodynamic investigation of colistin against <i>Pseudomonas aeruginosa</i> using an <i>in vitro</i> model	Published	70%
5	Clinically relevant plasma concentrations of colistin in combination with imipenem enhance pharmacodynamic activity against multidrug-resistant <i>P. aeruginosa</i> at multiple inocula	Accepted	70%
6	Synergistic killing of multidrug-resistant <i>Pseudomonas aeruginosa</i> at multiple inocula by colistin combined with doripenem in an <i>in vitro</i> PK/PD model	Accepted	80%

For each published or accepted manuscript, sections have been renumbered and references, abbreviations, nomenclature and writing conventions have been reformatted in order to generate a consistent presentation within this thesis. Some graphs published in black and white have been reproduced in colour for clarity.

Phillip J. Bergen

September, 2011

Acknowledgements

This thesis is first and foremost dedicated to my LORD and saviour, Jesus Christ. It is my prayer that all that is contained within will bring glory and honour to Him.

To my beautiful wife, companion, and closest friend Sarah whose patience, love, dedication and understanding throughout the last seven years has been unfailing. Sweetheart, without you by my side completing this enormous task would never have been possible. And to my adorable children, Timothy and James, who have only ever really know Daddy to be a PhD student - thank you for your hugs, smiles, kisses and laughter which have lifted my spirits at the end of many a long day. I hope to spend more time with you in the future.

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While not directly involved in my PhD, I wish to thank the members of the Department of Pharmacy Practice for their friendship and support throughout this time. To Associate Professor Kay Stewart, whose assistance on academic matters was, unknowingly to her, incredibly important and greatly assisted in allowing me time to undertake my research, and to Mr (soon to be Dr) Kevin Mc Namara and Dr Safeera Hussainy, who are wonderful friends, I offer my sincere thanks.

To my loving parents, who have been wonderful role models and mentors throughout my life, and who have instilled in me a love of knowledge and, most importantly, a love of Jesus Christ – I give you my eternal thanks.

I have been truly blessed.

Publications

This thesis is a compilation of the following manuscripts:

- Chapter 1:** Bergen, P.J., J. Li, and R. L. Nation. 2011. Dosing of colistin – back to basic PK/PD. *Curr Opin Pharmacol* Aug 9. [doi: 10.1016/j.coph.2011.07.004]. Parts of Chapter 1 only.
- Chapter 2:** Bergen, P. J., J. Li, C. R. Rayner, and R.L. Nation. 2006. Colistin methanesulphonate is an inactive prodrug of colistin against *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **50**:1953-8.
- Chapter 3:** Bergen, P. J., J. Li, R. L. Nation, J. D. Turnidge, K. Coulthard, and R. W. Milne. 2008. Comparison of once-, twice- and thrice-daily dosing of colistin on antibacterial effect and emergence of resistance: studies with *Pseudomonas aeruginosa* in an *in vitro* pharmacodynamic model. *J Antimicrob Chemother* **61**:636-42.
- Chapter 4:** Bergen, P. J., J. B. Bulitta, A. Forrest, B. T. Tsuji, J. Li, and R. L. Nation. 2010. Pharmacokinetic/Pharmacodynamic investigation of colistin against *Pseudomonas aeruginosa* using an *in vitro* model. *Antimicrob Agents Chemother* **54**:3783-9.
- Chapter 5:** Bergen, P. J., A. Forrest, J. B. Bulitta, B. T. Tsuji, H. E. Sidjabat, D. L. Paterson, J. Li, and R. L. Nation. 2011. Clinically relevant plasma concentrations of colistin in combination with imipenem enhance pharmacodynamic activity against multidrug-resistant *P. aeruginosa* at multiple inocula. *Antimicrob Agents Chemother* Aug 29. [doi: 10.1128/AAC.05028-11]
- Chapter 6:** Bergen, P. J., B. T. Tsuji, J. B. Bulitta, A. Forrest, J. Jacob, H. E. Sidjabat, D. L. Paterson, R. L. Nation, and J. Li. 2011. Synergistic killing of multidrug-resistant *Pseudomonas aeruginosa* at multiple inocula by colistin combined with doripenem in an *in vitro* PK/PD model. *Antimicrob Agents Chemother* Sept 3. [doi: 10.1128/AAC.05298-11]

Presentations

The work in this thesis has resulted in the following presentations at national and international conferences:

1. **Bergen, P. J.**, J. Li, C. R. Rayner, and R. L. Nation. 2006. Colistin methanesulphonate is an inactive prodrug of colistin. The 7th Australian Society of Antimicrobials Meeting, Sydney, Australia (poster presentation).
2. **Bergen, P. J.**, J. Li, R. L. Nation, J. D. Turnidge, K. Coulthard, and R. W. Milne. 2007. Is once daily dosing best for colistin against *Pseudomonas aeruginosa*? The 8th Australian Society of Antimicrobials Meeting, Melbourne, Australia (oral presentation).
3. **Bergen, P. J.**, J. Li, R. L. Nation, J. D. Turnidge, K. Coulthard, and R. W. Milne. 2007. Comparison of once-, twice- and thrice-daily dosing of colistin on antibacterial effect and emergence of resistance: studies with *Pseudomonas aeruginosa* in an *in vitro* pharmacodynamic model. The 8th Australian Society of Antimicrobials Meeting, Melbourne, Australia (poster presentation).
4. **Bergen, P. J.**, C. Lacza, J. Li, A. Forrest, and R. L. Nation. 2007. An improved approach to pre-clinical modeling. The 17th International Society of Anti-infective Pharmacology (ISAP) Meeting, Chicago, USA (oral presentation).
5. **Bergen, P. J.**, J. B. Bulitta, A. Forrest, J. Li, and R. L. Nation. 2008. A pharmacokinetic/pharmacodynamic investigation of colistin against *Pseudomonas aeruginosa* using an *in vitro* model. Abstract A-1671. The 48th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC)/46th Infectious Disease Society of America (IDSA) Annual Meeting, Washington DC, USA (oral presentation).
6. **Bergen, P. J.**, J. B. Bulitta, A. Forrest, J. Li, and R. L. Nation. 2009. Pharmacokinetic/pharmacodynamic determinant of colistin activity against *Pseudomonas aeruginosa*. The 10th Australian Society of Antimicrobials Meeting, Melbourne, Australia (oral presentation).

7. **Bergen, P. J.**, J. B. Bulitta, B. T. Tsuji, A. Forrest, R. L. Nation, and J. Li. 2009. *In vitro* pharmacodynamics of the combination of colistin and imipenem against multidrug-resistant *Pseudomonas aeruginosa* at multiple inocula. Abstract A1-575. The 49th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) Annual Meeting, San Francisco, CA, USA (poster presentation).
8. Bulitta, J. B., J. Li, **P. J. Bergen**, A. Poudyal, H. H. Yu, R. J. Owen, B. T. Tsuji, R. L. Nation, and A. Forrest. 2009. New mechanism-based models linking receptor binding with bacterial responses for optimising antimicrobial drug development and therapy. The 7th Annual Congress of International Drug Discovery Science and Technology (IDDST), Shanghai, China (oral presentation).
9. Bulitta, J. B., **P. J. Bergen**, B. T. Tsuji, J. Li, R. L. Nation, and A. Forrest. 2009. Population pharmacodynamic modeling of the mechanism of action and emergence of sub-populations of *Pseudomonas aeruginosa* for a wide range of colistin dosage regimens. American Conference on Pharmacometrics, Mashantucket, CT, USA (poster presentation).
10. **Bergen, P. J.**, B. T. Tsuji, J. B. Bulitta, A. Forrest, J. Li, and R. L. Nation. 2010. Synergistic killing of multidrug-resistant *Pseudomonas aeruginosa* at multiple inocula by colistin combined with doripenem in an *in vitro* pharmacokinetic/pharmacodynamic model. Abstract A1-659. The 50th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) Annual Meeting, Boston, MA, USA (poster presentation).
11. Bulitta, J. B., **P. J. Bergen**, C. H. Tan, B. T. Tsuji, A. Forrest, A. Poudyal, H. H. Yu, C. Ku, R. Nightingale, K. Davis, R. L. Nation, and J. Li. 2010. Translational combination modeling of the synergy of colistin with rifampicin Against *Acinetobacter baumannii* and of colistin with doripenem against *Pseudomonas aeruginosa*. Abstract A1-660. The 50th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) Annual Meeting, Boston, MA, USA (poster presentation).

Glossary of abbreviations

AAG	α_1 -acid glycoprotein
Ara4N	4-amino-4-deoxy-L-arabinose
ATCC	American Type Culture Collection
AUBC	area under the killing curve
AUC	area under the concentration-time curve
AUCPAP	area under the population analysis profile curve
BAL	bronchoalveolar lavage
CBA	colistin base activity
CF	cystic fibrosis
cfu or CFU	colony forming units
CL	total body clearance
CL _R	renal clearance
CLSI	Clinical and Laboratory Standards Institute
C _{max}	maximum concentration
C _{max,SS}	steady-state maximum concentration
C _{min}	minimum concentration
C _{min,SS}	steady state minimum concentration
CMS	sodium colistin methanesulphonate
CNS	central nervous system
CrCL	creatinine clearance
CRRT	continuous renal replacement therapy
CSF	cerebrospinal fluid
C _{ss,avg}	average steady-state plasma concentration
CVVHDF	continuous venovenous hemodiafiltration

Dab	diaminobutyric acid
ESBL	extended-spectrum β -lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
<i>f</i>	prefix for free (unbound)
<i>f_u</i>	unbound fraction
FIC	fractional inhibitory concentration
g	gram
GlcN	glucosamine
h	hour
HD	intermittent hemodialysis
HPLC	high-performance liquid chromatography
IDSA	Infectious Disease Society of America
IM	intramuscular
IP	intraperitoneal
IU	international units
IV	intravenous
Kdo	3-deoxy-D- <i>manno</i> -oct-2-ulosonic acid
kg	kilogram
L	litre
LC-MS/MS	liquid chromatography coupled with tandem mass spectrometry
Leu	leucine
LPS	lipopolysaccharide
m	meter
MDR	multidrug-resistant
mg	milligram
MIC	minimum inhibitory concentration

min	minute
mL	millilitre
PAE	post-antibiotic effect
PAPs	population analysis profiles
PD	pharmacodynamic
PK	pharmacokinetic
SC	subcutaneous
SD	standard deviation
$t_{1/2}$	half-life
Thr	threonine
VAP	ventilator-associated pneumonia

Chapter One

General Introduction

1.1 Bad bugs, no drugs

Infectious diseases have been an important cause of morbidity and mortality throughout history. With the antibiotic era came a growing confidence that the threat to human health posed by microorganisms would disappear. However, resistance to the newly introduced antimicrobial agents emerged quickly and by the late 1940s some hospitals were already reporting penicillin resistance rates of 85% for *Staphylococcus aureus* (1). This phenomenon persisted despite the subsequent introduction of various new categories of antimicrobial agents and today we face increasing resistance among Gram-positive and Gram-negative pathogens in both the hospital and community settings (Figure 1-1) (2-4). While new antibiotics active against Gram-positive organisms have recently been approved (5-8), the situation is quite different for multidrug-resistant (MDR) Gram-negative bacteria.

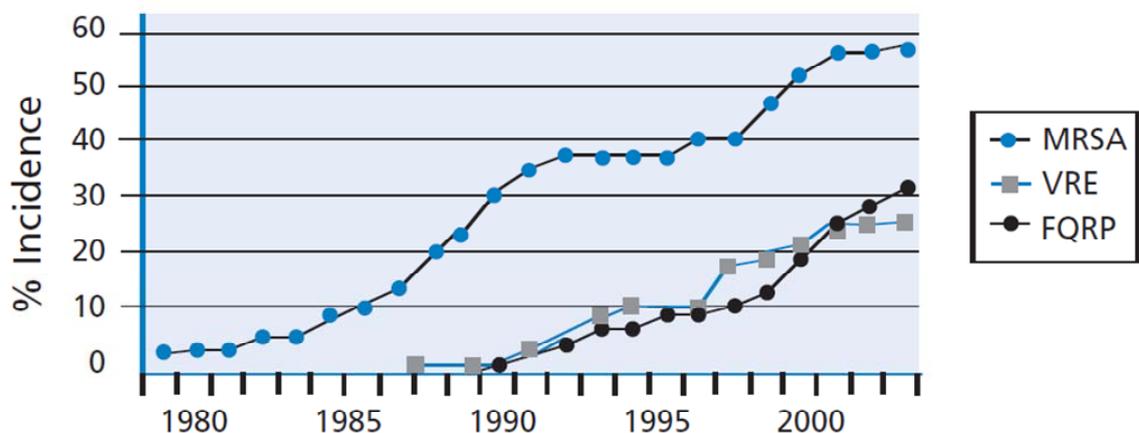


Figure 1-1. Increase in rates of resistance for three types of bacteria of public health concern: methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE), and fluoroquinolone-resistant *Pseudomonas aeruginosa* (FQRP). Figure reproduced from Infectious Disease Society of America (9), with permission.

Several highly resistant Gram-negative microorganisms – namely MDR *Pseudomonas aeruginosa*, *Acinetobacter* species, carbapenem-resistant *Klebsiella* species and *Escherichia coli* – are

emerging as significant pathogens worldwide (10). Therapeutic options for these pathogens are extremely limited, a situation made worse by the 'drying up' of the pharmaceutical development pipeline for anti-infective agents (Figure 1-2) (10-12). With an increasing immunocompromised population and an impressive ability of bacterial pathogens to adapt to and overcome the challenges of antibiotics, we are now faced with a growing population of MDR bacteria that threaten to move us into a 'postantibiotic era' of infectious diseases.

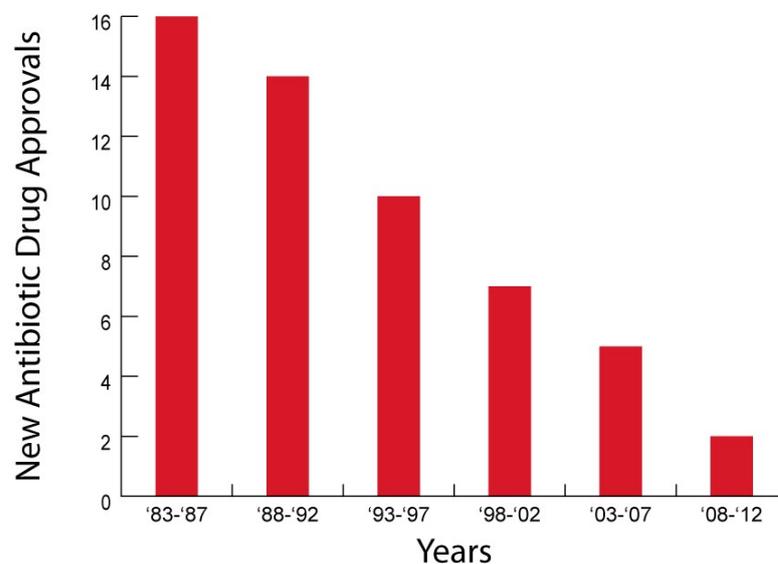


Figure 1-2. Number of new antibacterial agents approved in the United States, 1983 – 2011, per 5-year period. Figure adapted from Spellberg *et al.* (12), with permission.

P. aeruginosa has been identified by the Infectious Diseases Society of America (IDSA) as one of the top six pathogens threatening healthcare systems (10, 13). A versatile pathogen with the ability to cause diverse infection types, it is of central importance in a broad range of nosocomial and community-acquired infections including endocarditis, bone and joint infections, ophthalmologic disease, skin and soft tissue infections and, prominently, bloodstream infections and infections of the upper and lower airways (including ventilator-associated pneumonia [VAP]) (14-17). *P. aeruginosa* infections are responsible for increased lengths of hospital stay, need for surgical intervention, severe illness, death, and increased cost (18-21). While the overall

proportion of nosocomial infections caused by *P. aeruginosa* has remained stable over recent decades, the proportion of resistant isolates has increased alarmingly, particularly among critically-ill patients (19, 22-27). In one ICU surveillance study examining ~14,000 isolates of *P. aeruginosa*, rates of MDR isolates increased from 4% in 1993 to 14% in 2002 (Figure 1-3) (26). MDR *P. aeruginosa* is associated with significant morbidity and mortality (20, 25, 28-31), contributing to the poor prognoses associated with *P. aeruginosa* infections once established (32).

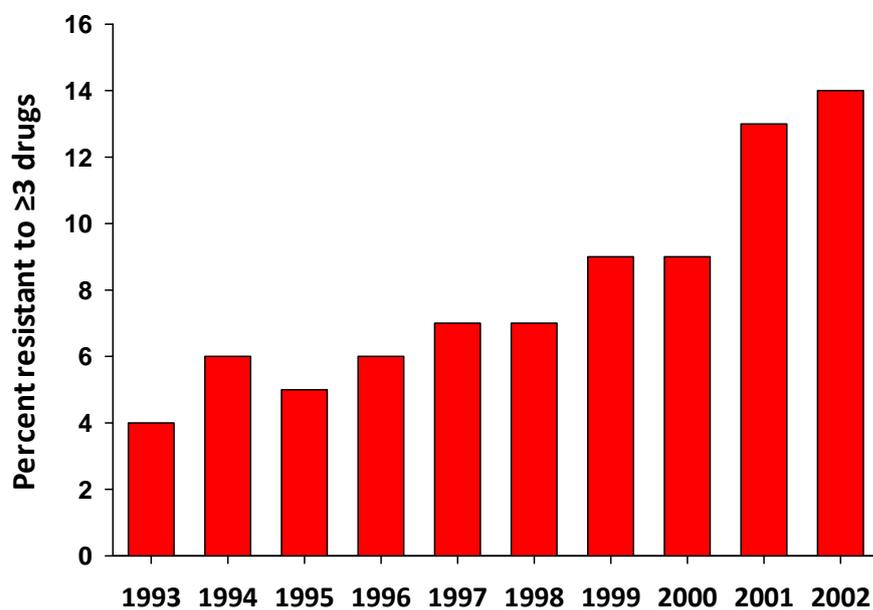


Figure 1-3. Increasing prevalence of multidrug resistance among *P. aeruginosa* isolates from ICU patients in the United States. Data for 13,999 nonduplicate isolates collected from 1993 to 2002 (26). Data represent the percentage of *P. aeruginosa* isolates that expressed a phenotype of multidrug resistance (defined as resistance to ≥3 drug classes) during each year of the study.

P. aeruginosa is intrinsically resistant to a wide array of antibacterials including many β -lactams, older fluoroquinolones, tetracyclines, macrolides, chloramphenicol, rifampicin and cotrimoxazole (trimethoprim/sulfamethoxazole) (14, 33). Antibacterial agents traditionally considered active against *P. aeruginosa* are shown in Table 1-1. However, *P. aeruginosa* is

capable of developing resistance to any of these agents, often under the influence of previous antibacterial exposure (14). Although the IDSA has placed *P. aeruginosa* on a 'hit list' of dangerous microorganisms requiring the most urgent attention for discovery of novel antibiotics, the availability of a new class of antibiotic able to evade the known resistance mechanisms of *P. aeruginosa* is many years away (11). Therefore, the challenge facing us today is to prevent or slow the emergence of resistance through optimising therapy with currently available drugs.

Table 1-1. Antibacterial agents with activity against *P. aeruginosa*.*

Antibiotic class	Drug	Mechanism of action
Penicillins	piperacillin and ticarcillin; includes the subgroups of β -lactam/ β -lactamase inhibitor combinations of ticarcillin/clavulanic acid and piperacillin/tazobactam	inhibit bacterial cell wall synthesis
Cephalosporins	cefepime, ceftazidime, cefsulodin, cefoperazone, cefpirome	inhibit bacterial cell wall synthesis
Carbapenems	imipenem/cilastatin, meropenem, doripenem (not ertapenem)	inhibit bacterial cell wall synthesis
Monobactams	aztreonam	inhibit bacterial cell wall synthesis
Fluoroquinolones	ciprofloxacin, moxifloxacin, levofloxacin, norfloxacin (urinary isolates only)	block DNA synthesis
Aminoglycosides	gentamicin, tobramycin, amikacin, netilmicin	inhibition of protein synthesis
Polymyxins	colistin, polymyxin B	disrupts the outer cell membrane; likely have additional intracellular targets

*Table compiled from references 34 – 36.

While there is an urgent need for new antibiotics, particularly those active against Gram-negative 'superbugs' (4, 11-13, 37), colistin (also known as polymyxin E), an old antibiotic previously abandoned because of toxicity concerns but which retains significant activity against these MDR Gram-negative microorganisms, is increasingly being used as an agent of last resort to treat infections caused by such bacteria (10, 38-42); in many cases, these pathogens are

susceptible only to colistin (13, 38-39, 43-47). This thesis will focus on increasing our understanding of the pharmacodynamics (PD) and pharmacokinetic/pharmacodynamic (PK/PD) relationships of colistin to facilitate optimisation of its clinical use in treating infections caused by *P. aeruginosa*.

1.2 Colistin

1.2.1 History of the polymyxins

The polymyxins were identified as products of the bacterium *Paenibacillus polymyxa* (previously known as *Bacillus polymyxa*) by two laboratories in the United States in 1947 (48-49). At the same time they were described as ‘aerosporins’ by a laboratory in Great Britain (50). By international agreement a nomenclature was developed that described the individual antibiotics as polymyxins A, B, C, D and E. Colistin was first described in 1950 (51) and originally thought to be distinct from polymyxins; however, it was subsequently determined to be identical to polymyxin E (52). Colistin is nonribosomally synthesized by *Pa. polymyxa* subspecies *colistinus* (51). Colistin and polymyxin B are the only two polymyxins used clinically.

Colistin, the focus of this thesis, has been available in the clinic since 1959 for the treatment of infections caused by Gram-negative bacteria (53). When early clinical reports suggested a high incidence of nephrotoxicity and neurotoxicity (54-56), its use declined with the arrival of the potentially less toxic aminoglycosides and other anti-pseudomonal agents. However, with the emergence of MDR Gram-negative bacteria resistant to almost all other available antibiotics (13, 57-58), and a lack of novel antimicrobial agents in the drug development pipeline for Gram-negative infections (4, 13, 58-59), the place of colistin in therapy is being re-evaluated.

There is little, if any, historical precedent for the re-emergence of an antibiotic into general use after it had long been relegated to the ‘back shelf’. Colistin was never subjected to the battery of

drug development procedures now mandated by international drug regulatory authorities, resulting in a lack of PK, PD, and other scientific information with which to inform rational use in order to maximise antibacterial activity and minimise toxicity and the development of resistance (39, 60). Critically, until recently there were no scientifically based dosage regimens for various categories of patients including subsets of critically-ill patients (e.g., with differing levels of renal function, including those on renal replacement therapy) and people with cystic fibrosis (CF). Worryingly, resistance to colistin (and polymyxin B) is beginning to emerge (45, 61-72); as there is only one amino acid difference between colistin and polymyxin B (Section 1.2.2) it is not surprising that cross-resistance is almost complete (60, 73-74). Given this alarming situation, there is an urgent need to optimise use of colistin to prolong its therapeutic utility.

1.2.2 Chemistry and commercial formulations

Colistin is a cationic, multi-component lipopeptide consisting of a cyclic heptapeptide ring with a tripeptide side chain acylated at the *N*-terminus by a fatty acid (Figure 1-4). Like most peptide antibiotics it contains a mixture of D- and L-amino acids and is characterised by a high percentage of 2,4-diaminobutyric acid (Dab) residues. At least thirty different components of colistin have been isolated (75-77), differing from each other by the composition of amino acids and fatty acids (75, 78). The two major components are colistin A (polymyxin E₁, N₂-(6-methyl-1-oxooctyl)-L-2,4-diaminobutanoyl-L-threonyl-L-2,4-diaminobutanoyl-L-2,4-diaminobutanoyl-L-2,4-diaminobutanoyl-D-leucyl-L-leucyl-L-2,4-diaminobutanoyl-L-2,4-diaminobutanoyl-L-threonyl-, cyclic (10→4)-peptide, C₅₃H₁₀₀N₁₆O₁₃) and colistin B (polymyxin E₂, N₂-(6-methyl-1-oxoheptyl)-L-2,4-diaminobutanoyl-L-threonyl-L-2,4-diaminobutanoyl-L-2,4-diaminobutanoyl-L-2,4-diaminobutanoyl-D-leucyl-L-leucyl-L-2,4-diaminobutanoyl-L-2,4-diaminobutanoyl-L-threonyl-, cyclic (10→4)-peptide, C₅₂H₉₈N₁₆O₁₃) (75-76) (Figure 1-4), which account for more than 85% of colistin (79); colistin A and colistin B differ by only one carbon atom and two protons in the fatty acyl tail. Because of the biological origin of this antibiotic, the proportion of colistin A and colistin

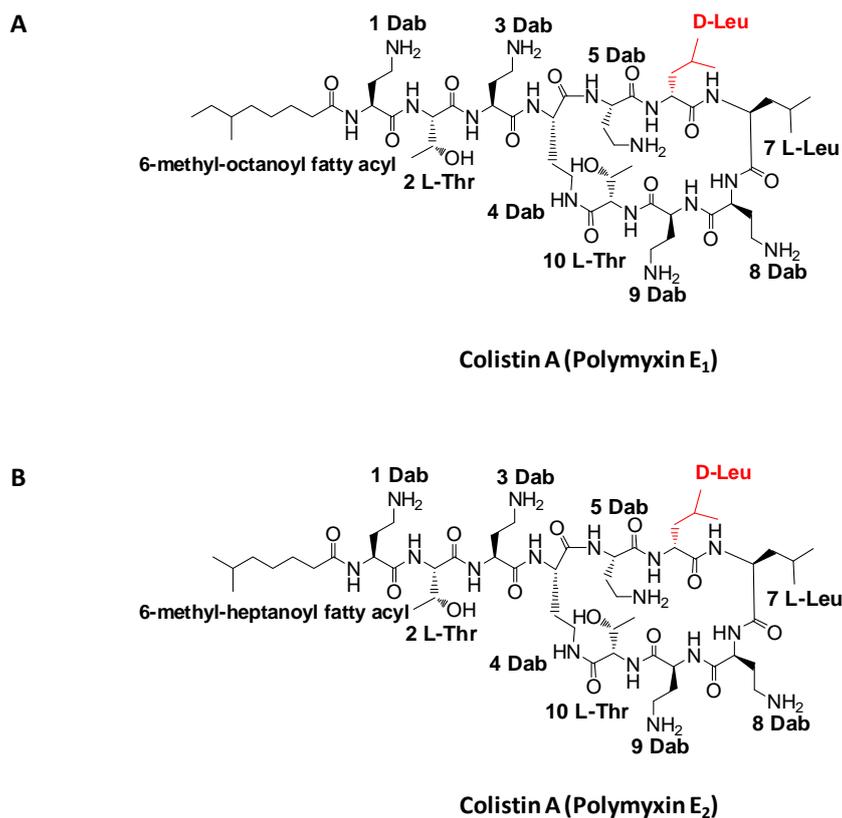


Figure 1-4. Molecular structures of colistin A (Panel A) and colistin B (Panel B). Fatty acid = 6-methyl-octanoic acid for colistin A, and 6-methyl-heptanoic acid for colistin B. Thr = threonine; Leu = leucine; Dab = 2,4-diaminobutyric acid. The D-Leu residue highlighted in red is substituted for D-Phe in the structure of polymyxin B.

colistin B in commercial products varies between manufacturers and batches (79-80). Other minor components include polymyxin E₃ and E₄ (81), norvaline-polymyxin E₁, valine-polymyxin E₁ (78), valine-polymyxin E₂, isoleucine-polymyxin E₁ (82), and polymyxin E₇ and isoleucine-polymyxin E₈ (75). The same primary amino-acid sequence to that of colistin is shared by polymyxin B, with a single substitution at position 6 in which the D-Leucine residue of colistin (highlighted in red in Figure 1-4) is replaced with D-Phenylalanine in polymyxin B. At physiological pH, the primary amines in the Dab residues (pK_a approximately 10) are ionised; thus, colistin is a cationic antimicrobial peptide (net charge of +5). Because colistin contains both polar (by virtue of the ionised Dab residues) and hydrophobic regions (particularly the fatty acyl tail), the

molecule is amphiphilic and able to distribute well in both polar and non-polar environments such as water and prokaryotic and eukaryotic lipid membranes (83). The cationic amphiphilic character is integral for the interaction of colistin with components of the outer membrane of Gram-negative bacteria, a key first step in the bactericidal action of this antibiotic (Section 1.2.6).

Two different forms of 'colistin' are available commercially: colistin sulphate (hereafter referred to as colistin), and sodium colistin methanesulphonate (CMS; also called colistimethate sodium, pentasodium colistimethanesulphate, and colistin sulphonyl methate) (Figure 1-5). Colistin is

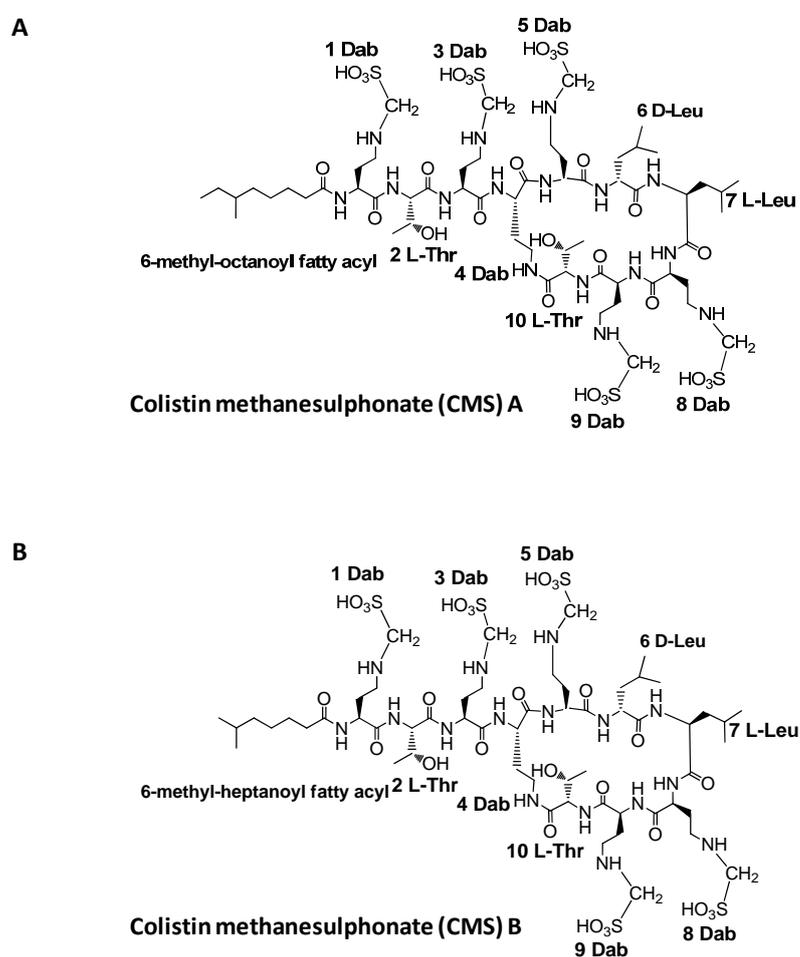
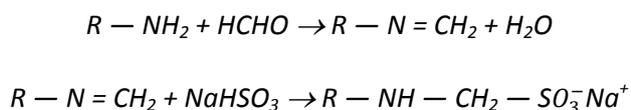


Figure 1-5. Molecular structures of colistin methanesulphonate (CMS) A (Panel A) and CMS B (Panel B). Fatty acid = 6-methyl-octanoic acid for CMS A, and 6-methyl-heptanoic acid for CMS B. Thr = threonine; Leu = leucine; Dab = 2,4-diaminobutyric acid.

poorly absorbed from the gastrointestinal tract and through skin (84) and is infrequently used orally for bowel decontamination and topically for the treatment of bacterial skin infections. CMS (present in parenteral and inhalational formulations in lyophilised form) is prepared from colistin by the reaction of the free 4-amino groups of the five Dab residues with formaldehyde followed by sodium bisulphite (85-86); this leads to the addition of a sulphomethyl group to each of the primary amines of colistin as follows:



CMS is less toxic than colistin when administered parenterally (86-87). Indeed, this was the reason for its development owing to concerns in early studies about the relatively high toxicity associated with parenteral administration of colistin (sulphate). CMS is poorly absorbed from the adult gastrointestinal tract (88) and is therefore administered parenterally, most commonly intravenously (IV) (38, 60). However, it may also be administered intramuscularly (IM), intrathecally, intraventricularly, and via inhalation, the latter a common route of administration for patients with CF. Although CMS can be administered IM at the same doses as IV, IM administration is not commonly used in clinical practice because of variable absorption and severe pain at the injection site (89). It is important not to use the terms colistin and CMS interchangeably as the chemistry, toxicity and PK of these two entities differ substantially (discussed below and in Section 1.5).

CMS is a polyanion at physiological pH (net charge -5) and has the potential to undergo conversion in aqueous solutions to form a complex mixture (up to 32 different products) of partially sulphomethylated derivatives, including colistin (85-86, 90); the charges on its partially sulphomethylated derivatives range from -3 to +3 (-3 with four attached sulphomethyl groups,

+3 with one attached sulphomethyl group). By the use of high-performance liquid chromatography (HPLC) or liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) methods for separate quantification of CMS and colistin in biological fluids (Section 1.5), the formation of colistin *in vivo* has been demonstrated in patients (91-100) and rats (101-102) receiving parenteral CMS; both the administered CMS and the formed colistin circulate in plasma. The conversion of CMS to colistin also occurs in the solutions for administration to patients that are produced by reconstitution of the lyophilised powder in pharmaceutical products (103). The toxicity and PK of colistin and CMS are discussed in Sections 1.2.5.2 and 1.5.

1.2.3 Labelling and dosing of parenteral CMS

Two factors complicating the clinical use of CMS are confusing labelling and inconsistent dosage recommendations. While all parenteral formulations of colistin contain the sulphomethyl derivative, CMS, some product labels express the contents in international units (IU; there are ~12,500 IU/mg of CMS (104)), while others are labelled with 'colistin base activity' (CBA; based upon microbiological standardisation). For example, the two most common commercially available parenteral formulations of CMS are Colomycin[®] (Dumex-Alpha A/S, Copenhagen, Denmark) and Coly-Mycin[®] M Parenteral (Parkedale Pharmaceuticals, Rochester, MN, USA). Colomycin[®] is used predominantly in Europe, whereas Coly-Mycin[®] is primarily used in the United States and Canada. Colomycin[®] is labelled in IU (500,000 IU, 1 million IU, and 2 million IU per vial) (105), equating to ~40, 80, and 160 mg of sodium CMS in each vial size, respectively. For a patient over 60 kg and with normal renal function, the manufacturer recommends a dose of 1 – 2 million IU three times daily (to a maximum of 6 million IU per day), equivalent to ~240 – 480 mg sodium CMS per day. In contrast, Coly-Mycin[®] M Parenteral is labelled as containing '150 mg colistin base activity' per vial (106-107), although each vial actually contains ~400 mg sodium CMS (equivalent to about 5 million IU). For a patient with normal renal function, the manufacturer recommends a dose of 2.5 – 5 mg/kg of CBA per day in 2 to 4 divided doses,

equivalent to ~6.67 – 13.3 mg/kg sodium CMS per day for a 60 kg patient. This equates to a daily dose of sodium CMS of ~400 – 800 mg, almost double that of Colomycin[®].

As can be seen by this complexity, the use of a unified dosage unit would greatly benefit the discussion of colistin dosing. The origin of this major discrepancy is unknown, and calls to resolve this disparity in labelling and manufacturer-recommended daily doses have so far gone unheeded (39, 108). Tragically, the confusion surrounding CMS labelling recently resulted in the death of a patient in the United States (109). In that case, the physician ordered the dose as mg of CMS rather than as CBA, the usual method of expressing the dose in the United States. This went unrecognised by pharmacists and nurses and resulted in the patient receiving doses ~2.7-fold higher than intended. The patient subsequently developed acute renal failure and other complications that resulted in their death. Differences in labelling and dosage recommendations flow through to published reports involving CMS/colistin, making comparisons between studies difficult. Indeed, many reports contain insufficient information to ascertain the actual dose of CMS used. Furthermore, many authors inappropriately refer to CMS and colistin interchangeably, when in fact the former is a derivative of the latter rather than a different salt of colistin. This distinction is especially important for PK studies because, as will be shown in Chapter 2, CMS is an inactive prodrug of colistin.

1.2.4 Antimicrobial spectrum

Colistin and polymyxin B have essentially identical *in vitro* potencies (as measured by minimum inhibitory concentrations [MIC]) and spectrums of activity against the commonly encountered Gram-negative organisms responsible for MDR nosocomial infections (110). Colistin exhibits a narrow antibacterial spectrum of activity, mostly against common Gram-negative pathogens. Colistin retains excellent bactericidal activity against most common species of Gram-negative bacilli or coccobacilli including *P. aeruginosa*, *Acinetobacter* spp. and *Klebsiella* spp. (71, 110-

119), the organisms against which it is most commonly used clinically. However, resistance to colistin in these and other species is increasing (45, 61-64, 66-72, 120-122). Interestingly, colistin-resistant isolates of several key species have been shown to be more susceptible to other antibiotics than their colistin-susceptible counterparts (42, 69, 123). Worryingly, colistin heteroresistance (the presence of resistant subpopulations within an isolate that is susceptible based upon its MIC) has been reported in *A. baumannii* (124-126) and *K. pneumoniae* (64, 127); in Chapter 5 of this thesis, colistin heteroresistance is reported for the first time in *P. aeruginosa*. The significance of colistin heteroresistance is discussed further in Sections 1.3 and 1.7.

Colistin is also active against *Enterobacter* spp. (71, 111), *E. coli* (71, 87, 111, 114), *Salmonella* spp. (87, 111), *Shigella* spp. (87, 111), *Citrobacter* spp. (111), *Haemophilus* spp. (128), *Bordetella pertussis* (85), *Legionella* spp. (129) and most *Aeromonas* species except *Ae. jandaei* (*Ae. hydrophila* has inducible resistance) (130). Colistin has also been reported to be potentially active against several mycobacterial species including *Mycobacterium xenopi*, *M. intracellulare*, *M. tuberculosis*, *M. fortuitum*, and the rapidly growing, non-pathogenic species *M. phlei* and *M. smegmatis* (131). Activity against *Campylobacter* species (132-133) and *Stenotrophomonas maltophilia* (113, 134-136) is variable, while activity against *Bartonella* species is borderline (137-138).

Colistin is generally inactive against *Vibrio* spp. (139), *Providentia* spp. (111, 140), *Serratia* spp. (87, 111, 114, 140-141), *Proteus* spp. (87, 114, 140), *Morganella morganii* (142), *Helicobacter pylori* (143-144), *Neisseria* spp. (meningococci and gonococci) (87, 145), *Brucella* spp. (87), *Edwardsiella tarda* (146), *Burkholderia cepacia* complex (113, 147), *P. pseudomallei* (148) and *Moraxella catarrhalis* (145). Colistin has no significant activity against most Gram-positive bacteria, anaerobes, parasites or fungi (73, 87, 149-151).

In determining susceptibility to ‘colistin’, confusion has surrounded whether colistin (sulphate) or CMS (sodium) should be used in MIC testing. This issue is addressed in detail in Chapter 2, which demonstrates that CMS is inappropriate for MIC measurement. The current Clinical and Laboratory Standards Institute (CLSI) (152) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) (153) MIC interpretative standards for colistin (based on colistin sulphate) using the microbroth dilution method are shown in Table 1-2. Given the emerging clinical PD and PK data (see Sections 1.3 and 1.5), the appropriateness of these breakpoints within a clinical context remains to be determined.

Table 1-2. Current minimum inhibitory concentration (MIC) interpretative standards for colistin using microbroth dilution.

Organism	MIC Interpretive Standard (mg/L)*		
	S	I	R
CLSI†			
<i>P. aeruginosa</i>	≤2	4	≥8
<i>Acinetobacter</i> spp.	≤2		≥4
Other non- <i>Enterobacteriaceae</i>	≤2	4	≥8
EUCAST‡			
<i>Pseudomonas</i> spp.	≤4		>4
<i>Acinetobacter</i> spp.	≤2		>2
<i>Enterobacteriaceae</i>	≤2		>2

* S, susceptible; I, intermediate; R, resistant

† CLSI, Clinical and Laboratory Standards Institute

‡ EUCAST, European Committee on Antimicrobial Susceptibility Testing

1.2.5 Clinical uses and adverse effects

1.2.5.1 Clinical uses

The resurgence in the use of CMS began in the late 1980s when it was administered IV and/or by inhalation to manage infection or colonisation with *P. aeruginosa* in patients with CF (154). For chronic *P. aeruginosa* lung infections in CF patients, regular nebulised CMS is recommended to

avoid the development of acute pulmonary exacerbations (155), whereas IV CMS is reserved for treatment of acute exacerbations (99, 156-160). CMS is also increasingly used in the treatment of pneumonia (including VAP), where it is administered either IV (for treatment (161-170)) or via inhalation (treatment (170-182) and prevention (176, 183-185)), and for bacteremia, where it is generally administered in combination with one or more antibacterial agents (161-162, 164, 167-168, 186-188).

CMS is administered IV either alone (162, 169, 189-191), or in combination (96, 98, 192-193), for central nervous system (CNS) infections such as meningitis and ventriculitis caused by MDR Gram-negative organisms. However, concerns about poor penetration of CMS and formed colistin into the CNS (96, 98, 194-195), and reports of failure (196-197) or toxicity (e.g., nephrotoxicity) (198) with IV therapy alone, have resulted in CMS increasingly being administered directly into the cerebrospinal fluid (CSF; either intrathecally (198-203) or intraventricularly (203-206)); intrathecal or intraventricular administration is not approved by the FDA. IV CMS has been used in the treatment of various other infections such as urinary tract infections (162-164, 186-187), catheter-related infections (162-164, 186-187), surgical site, skin and soft tissue infections (161-164, 186-187, 207), tracheobronchitis (174, 187), intraabdominal infections (162-165, 186-187, 207), wound infections including diabetic foot infections (165, 167, 208), osteomyelitis (163, 207), otitis media (161-162), sinusitis (167, 186), mediastinitis (207) and arthritis (161). CMS has also been used to treat infective endocarditis (209-211).

1.2.5.2 Adverse effects

The most common adverse effects associated with parenteral administration of CMS are nephrotoxicity and neurotoxicity (60, 154, 212-213). Bronchoconstriction is the most serious potential adverse effect of inhaled CMS (214-217).

Nephrotoxicity: Recent studies in critically-ill patients report an incidence of nephrotoxicity ranging from 0 to 56% (97, 161-164, 166-169, 186-187, 218-239). The wide range of nephrotoxicity rates can be at least partially explained by different definitions of renal toxicity, with >5 definitions reported in one systematic review (240). However, studies which utilise the recently developed consensus definition for acute kidney injury, the RIFLE criteria (**R**isk, **I**njury, **F**ailure, **L**oss, and **E**nd-stage kidney disease) (241), report rates of acute kidney injury of ~30–55% with standard CMS dosages (224, 235, 237, 239). While nephrotoxicity is almost always reversible (163, 187, 218, 224, 236, 240), it is nevertheless a major dose-limiting adverse effect which impacts the optimal use of CMS/colistin (Section 1.6).

Neurotoxicity: Neurologic adverse effects including dizziness, weakness, polyneuropathy, facial and peripheral paresthesia, partial deafness, vertigo, visual disturbance, confusion, ataxia and neuromuscular blockade which can lead to respiratory failure and apnoea (38). The incidence of colistin-associated neurotoxicity reported in earlier literature was ~7% (38), however it is rarely reported in recent studies and when observed is usually mild, reversible, and appears in patients receiving prolonged treatment (159, 161, 167, 186-187, 236, 242); apnoea has been reported only rarely in the last 20 years (240, 243-244). Neurotoxicity may be underreported as many patients requiring colistin are sedated and under mechanical ventilation, making assessment difficult (47).

1.2.6 Mechanisms of antibacterial action and resistance

Most studies investigating the mechanism of antibacterial action of the polymyxins have used polymyxin B as the model compound. As colistin and polymyxin B are structurally very similar (Section 1.2.2) (43), and display a high degree of cross-resistance (60, 73-74, 110), they are believed to share the same mechanism of antibacterial action. References to the ‘polymyxins’ in this section thus apply to both colistin and polymyxin B. As will be discussed below, it is generally

accepted that the initial target of polymyxins against Gram-negative bacteria is the lipopolysaccharide (LPS) component of the outer membrane, initiated by electrostatic attraction to the anionic phosphate groups of lipid A, followed by interactions between the hydrophobic domains of the polymyxin and the fatty acyl chains of lipid A (245-246). Therefore, an understanding of the mechanism of action against these bacteria requires knowledge of the structure of the Gram-negative cell envelope, and in particular LPS.

Structure of the Gram-negative cell envelope and LPS: The Gram-negative bacterial cell envelope is made up of an inner (cytoplasmic) membrane, the periplasmic space containing peptidoglycan, and an outer membrane (Figure 1-6) (247). The cytoplasmic membrane comprises a phospholipid bilayer with various proteins and enzymes accommodated throughout (248). The outer membrane, of particular interest when examining the mechanism of antibacterial action of the polymyxins, consists of an asymmetrical bilayer with inner and outer leaflets (Figure 1-6). The inner leaflet consists of lipoproteins and phospholipids (phosphatidylethanolamine, phosphatidylglycerol and cardiolipin) while the outer, more variable leaflet is comprised principally of heterogeneous LPS while also encompassing phospholipids, porins, proteins and lipoproteins (249-250). The outer membrane is the first line of defence for Gram-negative bacteria and serves as a permeability barrier to the passage of antibiotics, enzymes, detergents, heavy metals and bile salts (250-253) while also assisting in maintaining cell rigidity and shape (254).

LPS, the principal component of the external leaflet of the asymmetrical Gram-negative outer membrane, is the initial target of polymyxins. It can be conceptually divided into three components (Figure 1-6): (i) lipid A, the hydrophobic component of which anchors the LPS molecule in the outer leaflet of the outer membrane; (ii) the core oligosaccharide, which can be

further divided into inner and outer core; and (iii) the O-antigen polysaccharide (254, 256). LPS is often referred to as endotoxin (257).

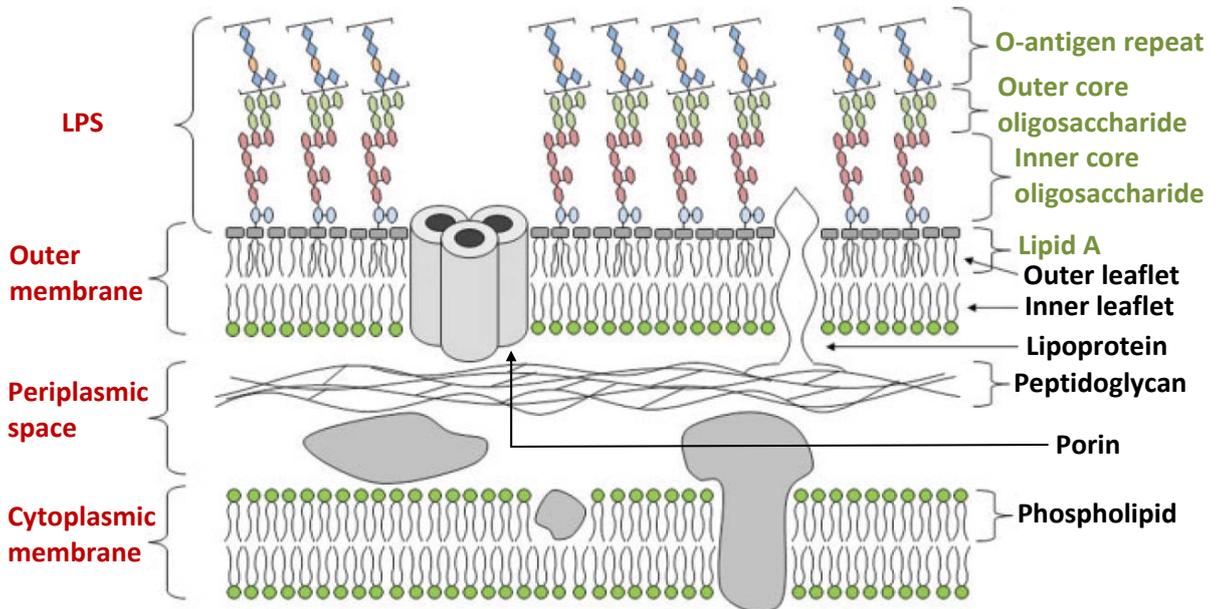


Figure 1-6. Structure of the Gram-negative cell envelope. Figure adapted from McEwen *et al.* (255), with permission.

Lipid A from Gram-negative species (including *P. aeruginosa*) is the domain of LPS which mediates inflammatory response-induced endotoxicity (258) and has a general structure consisting of a hexa-acylated di-glucosamine backbone which is phosphorylated at positions 1 and 4', and acylated with four 3-hydroxymyristic acid residues at positions 2, 3, 2' and 3' (259-260). The lipid A produced by most laboratory strains of *P. aeruginosa* is shown in Figure 1-7 (261). The anionic character of LPS is mainly afforded by carboxyl and phosphate moieties concentrated on lipid A, as well as sugars within the core oligosaccharide (257). The negatively-charged sites of LPS strongly bind divalent cations such as Mg^{2+} and Ca^{2+} , stabilising the outer membrane by non-covalently cross-bridging adjacent LPS molecules (73, 254, 262-266). The core oligosaccharide is directly linked to lipid A via Kdo (3-deoxy-D-manno-oct-2-

ulosonic acid). Among Gram-negative bacteria, *P. aeruginosa* has the most phosphorylated core with three phosphorylation sites (261, 267-269). Potential modifications of lipid A and core oligosaccharide leading to increased resistance to polymyxins are discussed below.

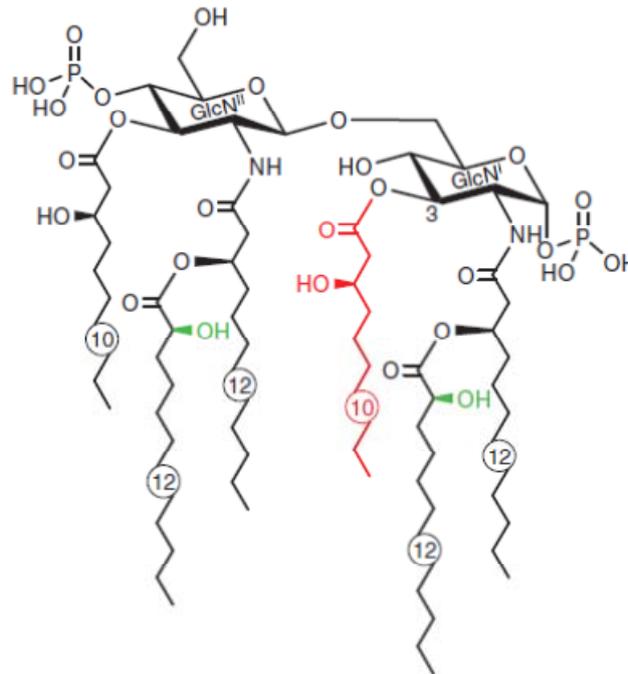


Figure 1-7. Typical lipid A produced by laboratory-adapted strains of *P. aeruginosa*. The 3-hydroxydecanoyl chain at the 3-position (red) is absent in approximately 75% of LPS molecules. Secondary acyl chains can exhibit 2-hydroxylation (green). GlcN = glucosamine. Figure reproduced from King *et al.* (261), with permission.

The O-antigens define the properties of the cell surface in species that do not produce capsules and are important in evading immune attacks from the host (254, 257, 270). Although lacking in some bacteria, LPS from most clinical specimens, including *P. aeruginosa*, contains an O-antigen domain (257, 261). The latter is a distinct repeating oligosaccharide which can extend up to forty units long, attached to a distal glucose residue of the core. O-antigen structures are highly variable compared to those of the core and lipid A (271).

Mechanism of action: The initial target of the polymyxins against Gram-negative bacteria is the LPS component of the outer membrane, initiated by electrostatic attraction between the cationic amine functionalities on polymyxin Dab amino acid residues and the anionic phosphate and carboxylate functionalities on the lipid A and core-oligosaccharide LPS domains (245, 249, 272-274). Polymyxins have an affinity for LPS at least three orders of magnitude higher than the native divalent cations (275-276), competitively displacing these cations (which function as a bridge between LPS molecules) from their LPS binding sites (73, 277-281). The bulkiness of the displacing polycations leads to a distortion of outer membrane structure (electron microscopy reveals this as blebbing (Figure 1-8) (282-286)) resulting in the formation of transient cracks through which ions and other low molecular weight polar molecules may permeate (245, 250, 264). Once electrostatically bound to LPS, the *N*-terminal fatty-acyl chain and D-Leu⁶-L-Leu⁷ (colistin) and D-Phe⁶-L-Leu⁷ (polymyxin B) hydrophobic domain is inserted into the lipid assembly of the outer membrane in a process driven by hydrophobic interactions, weakening the packing of adjacent lipid A fatty acyl chains and causing considerable disruption and further permeabilisation of the membrane, including to the peptide itself (264, 287-291). The polymyxins share this uptake mechanism with other cationic antimicrobial peptides, a process termed 'self-promoted uptake' by Hancock (Figure 1-9) (292). Changes in outer membrane permeability in the presence of polymyxins have been confirmed by the selective release of periplasmic proteins (73, 281, 293-296). The narrow spectrum of activity of polymyxins for Gram-negative bacteria is coincident with their binding selectivity for LPS (254, 265).

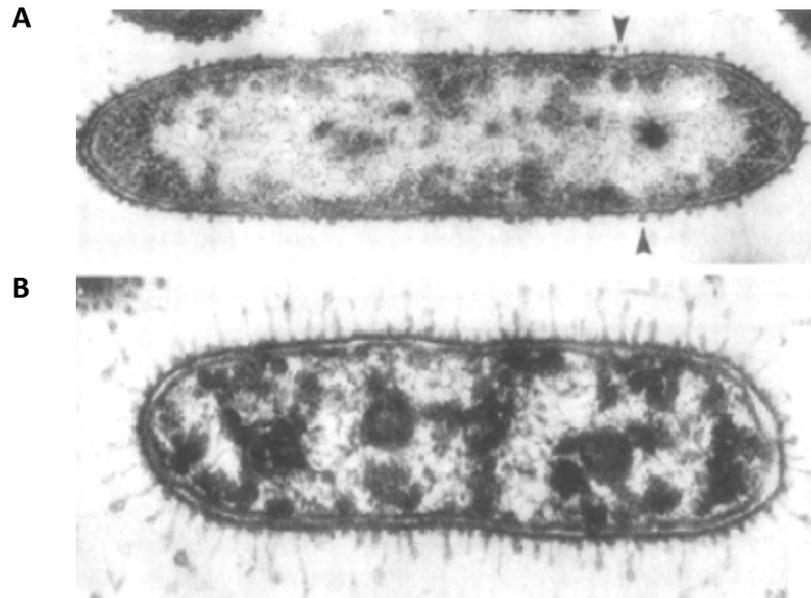


Figure 1-8. *P. aeruginosa* PAO1. (A) Typical healthy cell. Note the small blebs which arise from the outer membrane (arrows). (B) Cell grown in the presence of 6,000 IU/mL polymyxin B sulphate for 30 min. The cytoplasm appears clumped, and numerous long projections of outer membrane are seen. Image adapted from Gilleland and Murray (286), with permission.

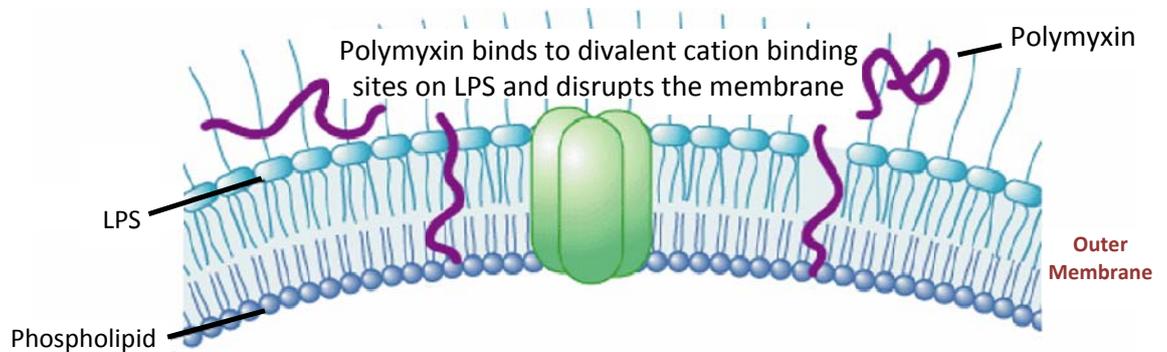


Figure 1-9. Proposed mechanism of self-promoted uptake of cationic antimicrobial peptides (including polymyxins) across the outer membrane of Gram-negative bacteria. These peptides bind to the negatively charged surface of the membrane and displace divalent cations, enabling hydrophobic regions to intercalate into the lipid A fatty-acyl region resulting in membrane disruption. Figure adapted from Hancock and Chapple (245), with permission.

Once the outer membrane has been breached, polymyxins enter the periplasmic space where they interact with anionic phospholipids of the cytoplasmic membrane in a process driven by electrostatic and hydrophobic interactions (272, 297-299). Under the influence of the large electrical potential of the bacterial cytoplasmic membrane, the cytoplasmic membrane is disrupted and permeability increased, resulting in membrane depolarisation and leakage of the cell contents (245, 274, 282-286, 291, 297, 300-302); disruption to both the outer and cytoplasmic membranes increases with increasing polymyxin concentration (282, 286). The large electrical potential and higher proportion of negatively-charged lipids on the surface monolayer of the bacterial cytoplasmic membrane plays an important role in the selectivity of antimicrobial peptides for bacterial cells over eukaryotic cells, which have low membrane potentials and predominantly uncharged lipids at the host cell surface (302).

It was originally proposed that permeabilisation of the bacterial cell membrane was the sole mode of action of cationic antimicrobial peptides, including the polymyxins, with insertion into the cytoplasmic membrane forming conductance events leading to leakage of cell contents and cell death (281, 298, 302-306). However, there is an increasing body of evidence that suggests that cationic antimicrobial peptides such as polymyxins exert their effects through an alternative mode of action, or that they may in fact act upon multiple bacterial cell targets (272, 297, 307-309). Polymyxins have been shown to induce rapid killing at concentrations considerably lower than that required for cytoplasmic membrane permeabilisation or depolarisation (264, 291, 310-311). Even at 200-fold the MIC, both colistin and polymyxin B caused minimal permeabilisation of the cytoplasmic membrane of *P. aeruginosa*, indicating that the actual bactericidal effects may not be related to membrane permeation (291). In addition, it has been shown that although polymyxin B and polymyxin B nonapeptide (i.e., polymyxin B minus the *N*-terminal fatty acyl chain and Dab¹ residue) cause similar perturbations of the *E. coli* cytoplasmic membrane at high concentrations, the latter has only weak antimicrobial activity (312). Recently, Pamp *et al.* (313)

found that an efflux pump (MexAB-OprM) may be involved in the development of resistance to colistin in *P. aeruginosa* when grown as a biofilm, indicating that colistin may also have an intracellular target. Thus, it appears that the bactericidal activity of the polymyxins may involve some event other than breakdown of the cytoplasmic membrane. In this way, their known abilities to act on lipid membranes may simply reflect their mechanism of passage across the membrane to their actual target site(s) in the cytoplasm.

Several alternative mechanisms of action for the polymyxins have been suggested. These include the loss of the compositional specificity of each membrane resulting in an osmotic imbalance in the absence of cell lysis or cytoplasmic leakage (291, 314-318), the arrest of cell proliferation (319), and inhibition of the NADH dehydrogenase family of enzymes involved in bacterial respiration (309). However, the precise mechanism(s) by which they act is still unknown and awaits further investigations.

Mechanisms of antibacterial resistance: Early investigations into the mechanisms of polymyxin resistance were conducted primarily in *P. aeruginosa* and revealed two distinct forms of resistance. Mutational resistance results from rare spontaneous mutations and involves a significant increase in outer membrane protein H1 with a concomitant decrease in the Mg^{2+} content of the cell envelope (278, 320-321). Adaptive resistance (also called adaptive tolerance (322) or phenotypic tolerance (313, 323)), a phenomenon by which certain environmental cues can transiently induce resistance to otherwise lethal doses of antimicrobials (324), is common and is differentiated from mutational resistance by its lack of inheritability and reversion to the susceptible phenotype upon removal of the inducer. Adaptive resistance to polymyxins is known to occur in response to limiting concentrations of divalent cations such as Mg^{2+} and Ca^{2+} (324-329) or stepwise exposure to increasing polymyxin concentrations (286, 330-331). Both mutational and adaptive resistance to the polymyxins is the result of a complex interplay of

several systems all of which appear to mediate resistance by influencing the ability of polymyxins to bind to, and hence permeabilise, the outer membrane; the cytoplasmic membrane of resistant organisms retains its sensitivity to disruption (332). The two forms of resistance are not mutually exclusive, with resistant mutants able to undergo further alterations of the outer membrane when grown in the presence of polymyxins, most likely the result of adaptive resistance mechanisms (320). Additionally, mutations in the regulatory pathways involved in adaptive resistance can lead to an inheritable, resistant phenotype (333-338).

Given that the crucial first step in the action of polymyxins on Gram-negative bacterial cells is the electrostatic interaction between the positively charged polymyxins and the negatively charged LPS, it is not surprising that resistance to polymyxins (and other cationic antimicrobial peptides) often involves modifications in LPS structure. These modifications, the nature and extent of which varies amongst different Gram-negative species due to differing constitutive lipid A modifying proteins, typically reduce the net negative charge on LPS and hence the initial electrostatic interaction between bacterium and polymyxin (339-340). As a consequence, binding to the outer membrane and subsequent self-promoted uptake is reduced or inhibited. Modifications to the lipid A and/or core of LPS with polar moieties such as 4-amino-4-deoxy-L-arabinose (Ara4N) and phosphoethanolamine have been observed in *P. aeruginosa* (327, 333, 341-343), *Escherichia coli* (344-347), *Salmonella typhimurium* (339, 344, 348-351), *K. pneumoniae* (352) and other bacteria (353-359). In many cases these changes have also been shown to increase resistance to polymyxins (333, 345, 348, 351, 353, 355-361). Several two-component regulators involved in LPS modifications in *P. aeruginosa* have been identified, including PhoP-PhoQ, PmrA-PmrB, and ParR-ParS (324, 333, 342, 362-364); analogous pathways exist in other Gram-negative bacteria (339, 348, 363, 365-368). Recently, colistin resistance in *A. baumannii* mediated by complete loss of LPS (due to mutations within genes of the lipid A biosynthetic pathway) was reported (369).

Clinical evidence that the outer membrane changes described above play a role in the development of polymyxin resistance is accumulating. *Pseudomonas* strains isolated from infants with CF had Ara4N and palmitate additions to lipid A as an early adaptation (327, 343), consistent with the notion that cationic antimicrobial peptides impose selective pressure on *P. aeruginosa* in the airways of individuals with CF (370); in the human host, *P. aeruginosa* is exposed to endogenous cationic antimicrobial peptides such as β -defensins (370-371) and cathelicidins (372). Colistin-resistant strains of *P. aeruginosa* isolated from patients with CF receiving inhaled CMS as routine maintenance therapy also have alterations in the lipid A structure, including the addition of Ara4N (120, 333, 373).

Recent reports have also revealed the repeated and independent emergence of colistin resistance in several bacterial species, including *P. aeruginosa* (69-70, 374). This suggests that colistin resistance may be acquired repeatedly, most probably due to selective pressure by increasing and suboptimal use of colistin; reports of polymyxin resistance arising during treatment of patients with polymyxins (both colistin and polymyxin B) are consistent with this hypothesis (124, 375). This emphasises the urgent need to optimise colistin dosage regimens to prevent the emergence and spread of colistin resistance.

1.3 Pharmacodynamics of colistin

General Pharmacodynamics: Antimicrobial PD is concerned with the relationship between concentration and antimicrobial effect (376). The major parameter used to quantify the activity of an antimicrobial agent against the infecting pathogen has traditionally been the MIC (376). The MIC, defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation, is a measure of the potency of an antimicrobial agent and varies depending on the agent and organism (110, 377). Current

measures used to assess the relationship between PK and PD of an anti-infective are referenced to the MIC, and are discussed in Section 1.4.

Although the MIC is a good predictor of the potency of the drug-organism interaction, it is determined at a fixed point in time after exposure to static drug concentrations and hence provides no information on the time course of antimicrobial activity or details of any postantibiotic effect (PAE); PAE refers to the persistent suppression of bacterial growth following exposure to an antimicrobial (378-380). The effect of increasing concentrations on antimicrobial activity and the magnitude of persistent effects give a much better description of the time course of antimicrobial activity than is provided by the MIC. Antibiotics are typically categorised into two major groups based on their patterns of killing: (i) those that exhibit concentration-dependent killing, which occurs over a wide range of concentrations with moderate to prolonged persistent effects (examples include the aminoglycosides and fluoroquinolones); and (ii) those that exhibit time-dependent killing, which occurs over a narrow range of concentrations (due to saturation of the killing rate at low concentrations) with minimal to moderate persistent effects (examples include the β -lactams and macrolides) (376, 381). Figure 1-10 illustrates the effect of increasing concentrations on the *in vitro* antimicrobial activity of three different classes of antimicrobials against a strain of *P. aeruginosa*. Increasing concentrations of tobramycin (an aminoglycoside) and ciprofloxacin (a fluoroquinolone) resulted in more rapid and extensive bacterial killing, whereas a similar effect for ticarcillin (a β -lactam) was only observed when the concentration was increased from 1 to 4 \times MIC; higher ticarcillin concentrations (4 to 64 \times MIC), although associated with an earlier initiation of bacterial killing, did not increase the rate of killing after 2 h of treatment (380). The PD characteristics described here are an important consideration when designing dosage regimens. This will be discussed further in Section 1.4.

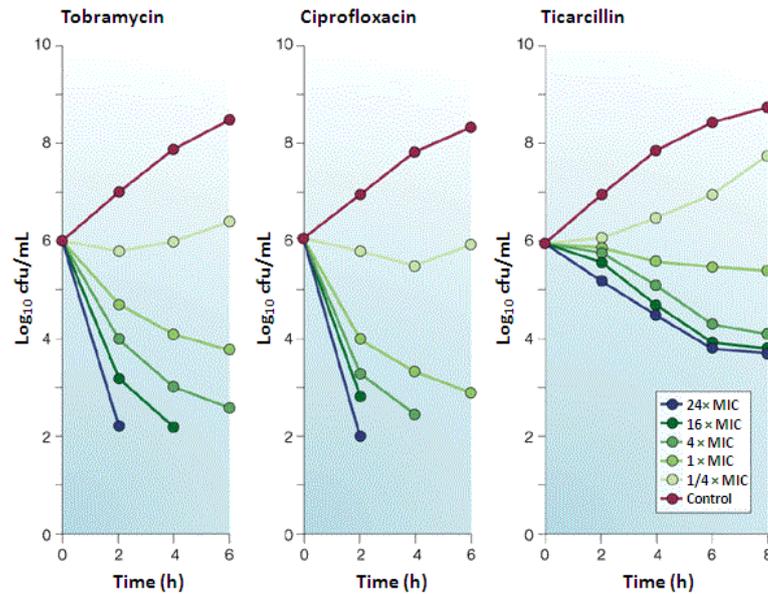


Figure 1-10. Bacterial killing against *P. aeruginosa* exposed to tobramycin (left), ciprofloxacin (centre), and ticarcillin (right) at concentrations from $\frac{1}{4}$ to 64x MIC. Data from Craig (380). Figure adapted from Drusano (382), with permission.

Pharmacodynamics of colistin: The current MIC interpretive standards for colistin are presented in Table 1-2 of Section 1.2.4. A recent study from the SENTRY Antimicrobial Surveillance Program (2006 – 2009) examined the antimicrobial activity of colistin and polymyxin B against 40,625 Gram-negative isolates (110). The MIC_{50} and MIC_{90} (MIC required to inhibit the growth of 50% and 90% of isolates, respectively) values for colistin for the bacterial species investigated in that study are presented in Table 1-3; values for polymyxin B were essentially identical to those of colistin. Only minor variations in the percentage of isolates susceptible to colistin were evident between distinct geographical regions, with 99.6, 99.5, 99.4, and 99.2 percent susceptible in 2009 in North America, Europe, Latin America and the Asia-Pacific, respectively. While the polymyxins presented excellent *in vitro* activity against the vast majority of the isolates evaluated, a trend to greater resistance was observed in regions where polymyxins have become more heavily prescribed.

Table 1-3. Antimicrobial activity of colistin against 40,625 Gram-negative isolates (SENTRY Antimicrobial Surveillance Program, 2006 – 2009). Antimicrobial susceptibility testing was performed and interpreted using the Clinical and Laboratory Standards Institute broth microdilution method except for Enterobacteriaceae, for which the European Committee on Antimicrobial Susceptibility Testing criteria was applied (152-153). Table adapted from Gales *et al.* (110), with permission.

Organism (number tested)	MIC (mg/L)	
	50%	90%
<i>P. aeruginosa</i> (9130)	1	1
<i>Acinetobacter</i> spp. (4686)	≤0.5	1
<i>Klebsiella</i> spp. (9774)	≤0.5	≤0.5
<i>E. coli</i> (17035)	≤0.5	≤0.5

Complicating the interpretation of MIC values reported for colistin is the phenomenon of colistin heteroresistance, the situation where colistin-resistant subpopulations are present within an isolate that is considered susceptible based upon MICs. As discussed in Section 1.2.4, colistin heteroresistance has been reported in *A. baumannii* (124-126) and *K. pneumoniae* (64, 127). This phenomenon is clearly evident in Figure 1-11, with colistin-resistant subpopulations detected in a majority of *K. pneumoniae* isolates considered susceptible based upon its MIC. Colistin heteroresistance in *P. aeruginosa* is reported for the first time in Chapter 5 of this thesis. It has not been established if heteroresistance reflects genetic heterogeneity in a mixed population or genetically identical cells that express different gene sets in response to divergent regulatory proteins (383-384). Heteroresistance contributes to regrowth following colistin therapy (discussed below), and provides a strong theoretical basis for colistin to be used as part of combination antimicrobial therapy to maximise antimicrobial activity (discussed in Section 1.7).

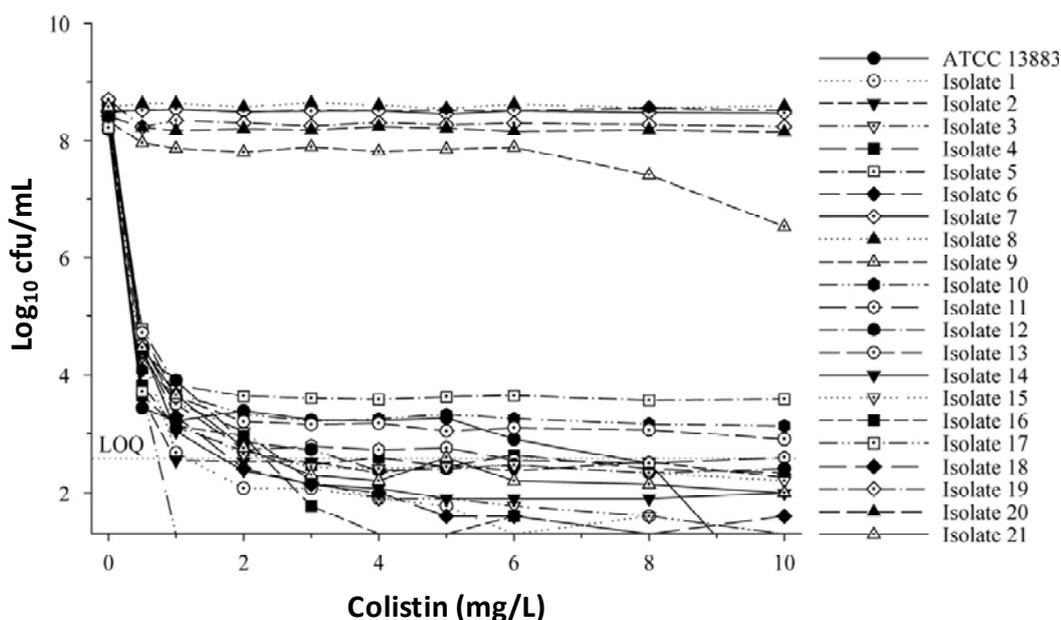


Figure 1-11. Population analysis profiles of 21 clinical isolates and a reference strain of *K. pneumoniae*. Resistant subpopulations (able to grow in the presence of >2 mg/L colistin) were observed in the reference strain and in 14 of 15 colistin-susceptible clinical isolates despite MICs between 0.125 and 1 mg/L. Figure reproduced from Poudyal *et al.* (127), with permission.

Most investigations examining the PD of polymyxins (colistin or polymyxin B) have focused on colistin and have been generated using *in vitro* models using either static or dynamic (PK/PD) time-kill methods (63, 74, 125-127, 385-388); time-kill methods are discussed further in Section 1.7.2.2. Given that colistin and polymyxin B are structurally very similar (Section 1.2.2), display a high degree of cross-resistance (60, 73-64, 110), and are believed to share the same mechanism of antibacterial action (Section 1.2.6), PD studies examining both colistin and polymyxin B are included here. While many early reports on antibacterial activity examined both colistin and CMS, Chapter 2 of this thesis will show that CMS is an inactive prodrug of colistin. Thus, activity reported with the use of CMS, whether from studies conducted *in vitro* or *in vivo*, derives from the formation of the active species, colistin.

Time-kill studies with colistin (sulphate) showed potent, concentration-dependent killing against *P. aeruginosa* (63, 385-388), *A. baumannii* (125-126) and *K. pneumoniae* (127), including MDR and, for *A. baumannii* and *K. pneumoniae*, heteroresistant strains (Figure 1-12). In the only study examining the PD of polymyxin B, similar concentration-dependent activity was observed against *P. aeruginosa* (Figure 1-13) (74). Initial killing for both polymyxins is very rapid. For colistin, a large decrease in colony forming units (cfu) per mL occurred as early as 5 min after antibiotic exposure to colistin concentrations in the vicinity of the MIC and above (63); similar decreases in cfu/mL were reported at 2 h (the time of the first sample) with polymyxin B (Figure 1-13) (74).

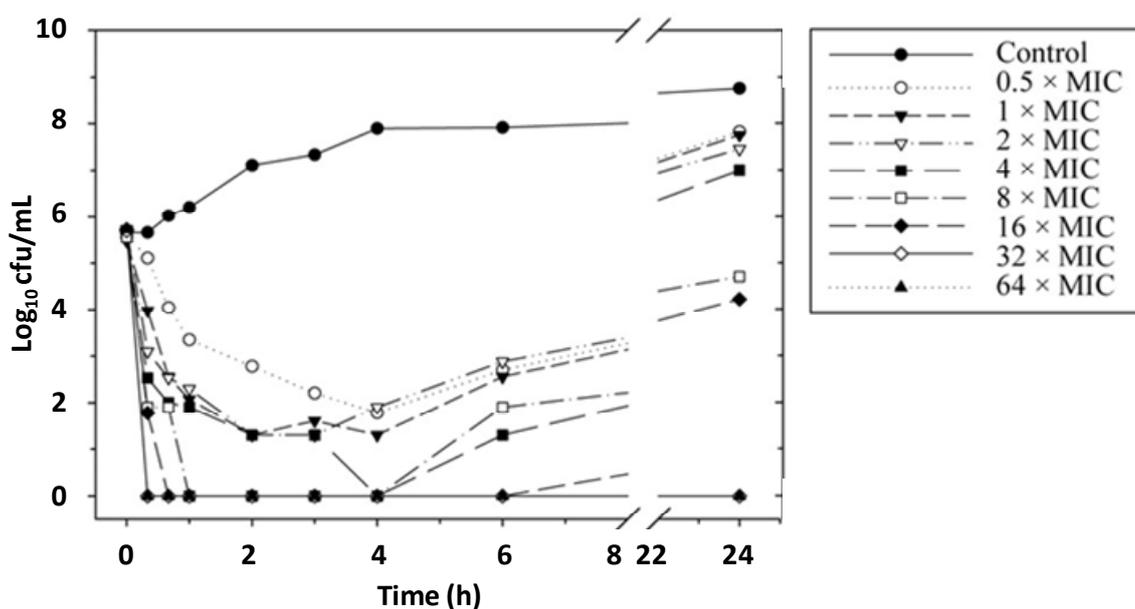


Figure 1-12. Time-kill studies with colistin against clinical isolates of *A. baumannii*. Figure reproduced from Owen *et al.* (125), with permission.

The antibacterial activity of both colistin and polymyxin B is subject to an inoculum effect (74, 385). Bulitta *et al.* (385) demonstrated both the rate and extent of killing by colistin is decreased at high compared to low inocula. Using a genetically characterised isolate of *P. aeruginosa* (PAO1), killing of the susceptible population at an inoculum of 10^9 or 10^8 cfu/mL was 23- and 6-fold slower, respectively, compared with an inoculum of 10^6 cfu/mL. At the 10^9 inoculum,

up to 32-fold higher concentrations were required to achieve bactericidal activity ($\geq 3\text{-log}_{10}$ cfu/mL decrease) compared with the 10^6 inoculum; mathematical modeling described the inoculum effect as a phenotypic change of bacterial cells. Similarly, Tam *et al.* (74) showed the rate and extent of killing by polymyxin B against 4 strains of *P. aeruginosa* was significantly reduced at an inoculum of 10^7 cfu/mL compared to 10^5 cfu/mL (Figure 1-13). Thus, there is a potential need for higher colistin exposure or combination regimens to treat deep-seated, difficult-to-treat infections with high inocula.

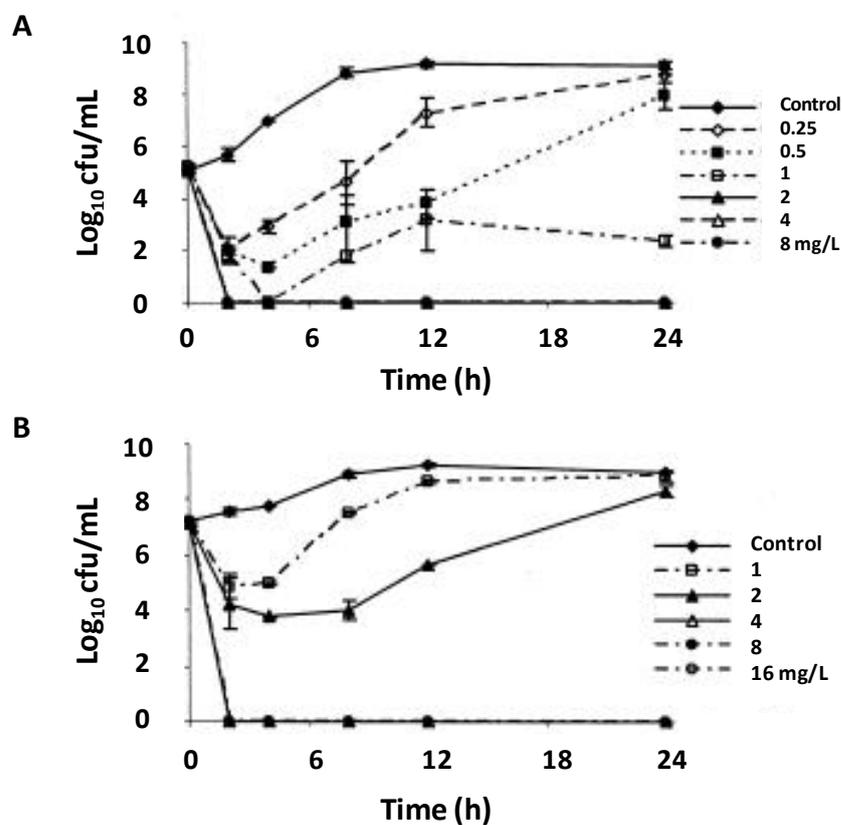


Figure 1-13. Time-kill studies with polymyxin B against a reference strain of *P. aeruginosa* (ATCC 27853) at an initial inoculum of 10^5 cfu/mL (Panel A) and 10^7 cfu/mL (Panel B). Concentration-dependent activity is evident, with bacterial killing reduced at the higher inoculum. Figure adapted from Tam *et al.* (74), with permission.

Colistin possesses at best a moderate PAE (63, 125, 127), a property which has significant implications for optimal dosing schedules. Against *P. aeruginosa*, colistin produced a significant PAE (>1 hour) only at high multiples (8× and 16×) of the MIC (Figure 1-14) (63). Similarly modest PAEs (1.0 – 3.5 h) were observed against *A. baumannii* with colistin concentrations $\geq 16\times$ MIC (125), while against *K. pneumoniae* a negligible or very modest PAE (1.6 h) was observed only at concentrations $\geq 64\times$ MIC (127); no studies have examined the PAE of polymyxin B. As will be discussed in Section 1.6, concentrations as low as 8× the MIC are unlikely to be achieved clinically for the majority of bacterial strains. Therefore, it appears unlikely that colistin would produce a PAE *in vivo*. However, although the product information for CMS recommends administration in two to four divided doses (106-107), CMS has recently been administered clinically using larger unit doses administered every 24 h, despite a lack of supporting PK/PD data (387, 389). Presumably larger, less frequent doses are to take advantage of the concentration-dependent killing exhibited by the polymyxins. However, by extending the dosage interval for CMS therapy, formed colistin concentrations would remain below the MIC of the organism for greater periods of the dosage interval. Given the likely lack of a PAE at clinically achievable concentrations, such a dosing strategy may not be appropriate as regrowth of the organism may commence soon after colistin concentrations fall below the MIC. This is discussed further in Section 1.4. The effect on antimicrobial activity and the emergence of colistin resistance from administering larger doses of CMS less frequently is the focus of Chapter 3. Finally, emerging toxicodynamic data in animals suggest larger, less frequent doses of colistin may increase nephrotoxicity (390); this is discussed in Section 1.6.

A consistent finding of both *in vitro* (74, 125-127, 387-388, 391-393) and *in vivo* (394-395) studies is regrowth with colistin monotherapy even with concentrations well in excess of those which can be safely achieved clinically. Gunderson *et al.* (387) reported regrowth by 24 h of two

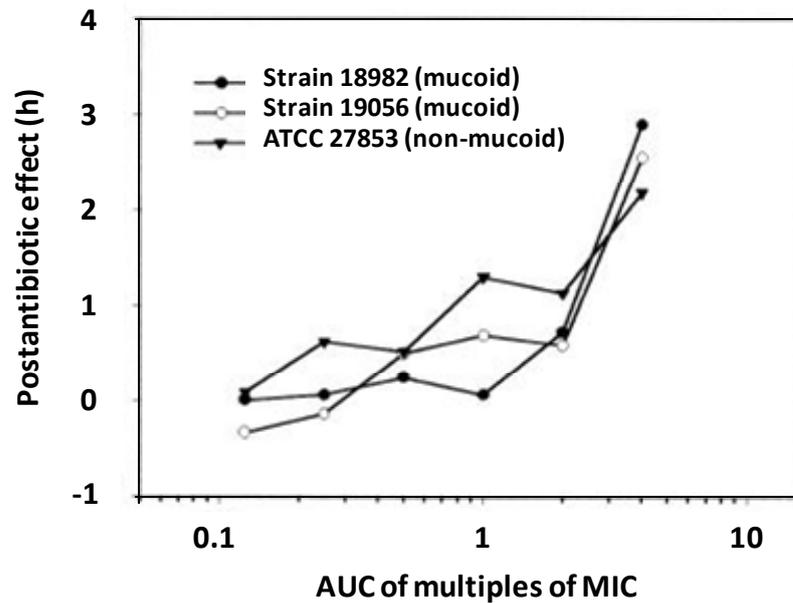


Figure 1-14. Post-antibiotic effects (PAE) of colistin against two clinical isolates and a reference strain of *P. aeruginosa*. Figure adapted from Li *et al.* (63), with permission.

MDR but colistin-susceptible clinical isolates of *P. aeruginosa* in an *in vitro* PK/PD model with maximal colistin concentrations (C_{max}) up to 200 mg/L (administered as single dose; range 0.5 – 200 mg/L) or 18 mg/L (12-hourly dosing; doses of 6 or 18 mg/L). Interestingly, colistin administered 12 hourly at a C_{max} of 18 mg/L produced fewer cfu/mL at 24 h (the duration of the experiment) than in any of the single-dose experiments. Similar findings were reported by Tam *et al.* (74) with polymyxin B in a hollow-fibre infection model with two strains (a wild-type strain and MDR clinical isolate) of *P. aeruginosa*. In that study, regrowth had occurred by 24 h in the majority of cases (and by 48 h in all cases) with polymyxin B administered 8-, 12-, or 24-hourly simulating the steady-state PK profiles of unbound polymyxin B resulting from a standard daily dose of 2.5 mg/kg; regrowth had returned close to control values with all regimens by 48 – 72 h. Regrowth with a higher simulated dose (20 mg/kg/day; 8 times the clinical dose) was

substantially reduced against the wild-type strain, but similar to standard doses with the MDR isolate. Employing a similar study design in a PK/PD model (but including a continuous infusion regimen), Tan *et al.* (126) examined colistin against two strains of *A. baumannii*. Regrowth occurred as early as 6 h, with extensive regrowth across the 6- to 24-h period to within $\sim 1 \log_{10}$ cfu/mL of the corresponding growth control. Similar regrowth of *A. baumannii* (125) and *K. pneumoniae* (127) following colistin monotherapy has been reported using static time-kill methodology with colistin concentrations up to 64× MIC.

Amplification of colistin-resistant subpopulations has been shown to contribute to the observed regrowth following colistin or polymyxin B monotherapy (74, 126-127, 385, 394). Early regrowth occurred in *in vitro* models utilising heteroresistant strains of *K. pneumoniae* (127) and *A. baumannii* (126) despite colistin concentrations well above the MIC, with population analysis profiles (PAPs) revealing substantial increases in the proportion of colistin-resistant subpopulations (Figure 1-15); these were substantially different from the PAPs prior to colistin therapy and those for the growth controls. A similar increase in the proportion of colistin-resistant bacteria was observed in murine thigh and lung infection models with heteroresistant *A. baumannii* (394). These observations suggest that the susceptible bacterial populations were selectively eradicated, resulting in unopposed growth of resistant subpopulations (such as LPS-deficient *A. baumannii* [Section 1.2.6] (369)) and consequently the emergence of resistance over time. Prior to work undertaken in Chapter 5 of this thesis, colistin heteroresistance had gone unreported for *P. aeruginosa*. However, increases in colistin- or polymyxin B-resistant subpopulations similar to those observed with heteroresistant *K. pneumoniae* (127) and *A. baumannii* (126) had been reported in *P. aeruginosa* following colistin (385) or polymyxin B (74) monotherapy, and are also reported in Chapter 3 of this thesis. Heteroresistance notwithstanding, adaptive resistance (Section 1.2.6) may also contribute to

regrowth as evidenced by reversion to the susceptible state following serial passaging on drug-free plates of one of three isolates in the study by Tam *et al.* (74).

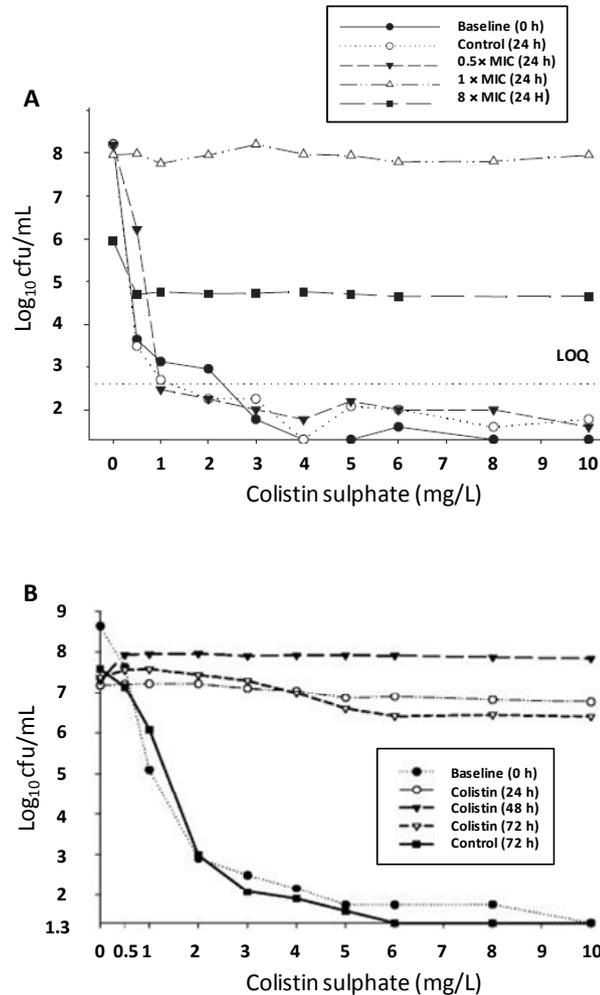


Figure 1-15. Changes in population analysis profiles (PAPs) for single colistin-heteroresistant clinical isolates of *K. pneumoniae* (Panel A) and *A. baumannii* (Panel B). *K. pneumoniae* was exposed to increasing concentrations of colistin over 24 h in a static time-kill model. *A. baumannii* was exposed to a colistin regimen simulating the unbound plasma concentration-time profile of colistin achieved with the recommended daily dosage regimen of CMS (5 mg/kg of body weight/day CBA), administered every 8 hours for 72 h in an *in vitro* dynamic model. Changes in PAPs clearly demonstrate the emergence of colistin resistance for both species. Figure adapted from Poudyal *et al.* (127) and Tan *et al.* (126), with permission.

The difficulty of eradicating colistin-resistant subpopulations with colistin monotherapy, together with the potential for rapid amplification of colistin-resistant subpopulations, suggests caution with the use of CMS monotherapy and highlights the importance of investigating rational colistin combinations. Use of CMS/colistin in combination with other antimicrobial agents is discussed in Section 1.7, and is the focus of Chapters 5 and 6.

1.4 Pharmacokinetic/pharmacodynamic driver of antibacterial activity

Interrelationship between PK and PD: The time course of antimicrobial activity is a reflection of the interrelationship between PK and PD and is an important determinant of effective dosage regimens (380, 382, 396-397). The PD characteristics described in Section 1.3 suggest that the time course of antimicrobial activity can vary markedly for different antibacterial agents. For example, the aminoglycosides and fluoroquinolones exhibit concentration-dependent killing and prolonged PAE; this suggests infrequent dosing of large doses should be possible because the prolonged PAE would protect against bacterial regrowth when concentrations fall below the MIC. The goal of a dosing regimen for these drugs would be to maximise their concentrations. As discussed in Section 1.3, recent reports of larger unit doses of CMS administered less frequently are presumably a response designed to take advantage of the concentration-dependent activity of the polymyxins observed *in vitro* (162, 387, 389). Conversely, β -lactams exhibit minimal concentration-dependent killing and generally produce a minimal to moderate PAE. Thus, the goal of a dosage regimen for these drugs would be to optimise the duration of exposure. Based on these considerations three different PK/PD indices have been described, with both the pattern of bactericidal activity and the presence or absence of PAE influencing which PK/PD index is the optimal driver of antimicrobial activity for a particular antimicrobial agent (398). The three indices are the maximal concentration to MIC ratio (C_{\max}/MIC), the area under the concentration-time curve over 24 h to MIC ratio (AUC/MIC), and the cumulative percentage of a

24-h period that concentrations exceed the MIC ($T_{>MIC}$); these are depicted in schematic form in Figure 1-16.

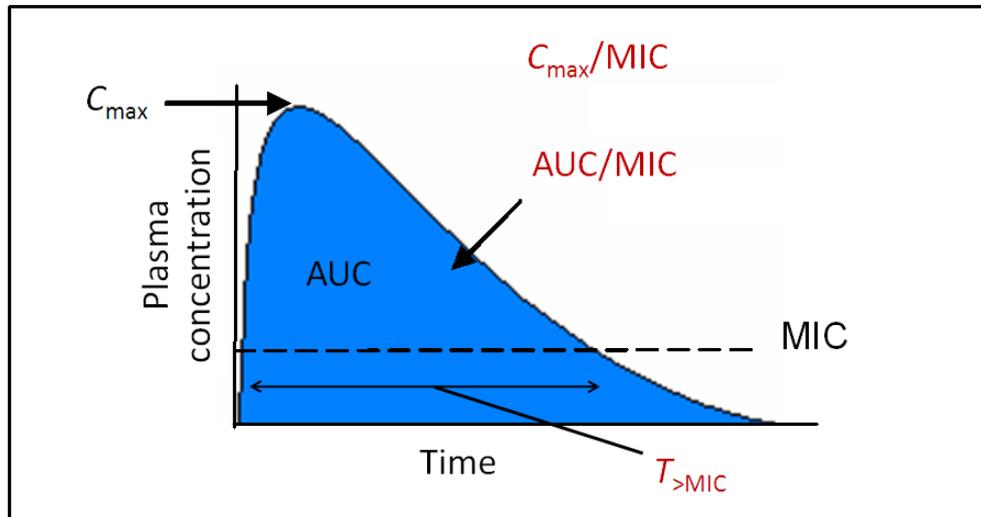


Figure 1-16. Relationship between the concentration-time profile, MIC and PK/PD indices (C_{max}/MIC , AUC/MIC and $T_{>MIC}$). It should be noted that the AUC/MIC and $T_{>MIC}$ are calculated over a 24-h period. Figure adapted from Kiem *et al.* (399), with permission.

Although there may be good theoretical grounds for predicting which PK/PD index is the best driver of antimicrobial activity in humans, it can be difficult to demonstrate in practice due to the design of clinical trials in which the efficacy of different dosage regimens is usually evaluated by comparing two or more doses of a drug administered at the same dosage interval. This can be seen in the left panel of Figure 1-17, where a fourfold-higher dose produces a higher C_{max}/MIC , higher AUC/MIC , and a greater $T_{>MIC}$. If the higher dose produces an enhanced therapeutic effect, it is not possible to determine which PK/PD index is of major importance as all three indices increased. To overcome this problem, dose fractionation (where the dosage regimen is varied and results compared) can be used to reduce the interdependence among the indices. As shown in the right panel of Figure 1-17, a dose administered every 2 h results in a lower

C_{\max}/MIC but a longer $T_{>\text{MIC}}$ than a fourfold-higher dose administered every 8 h; however, the AUC/MIC of the two regimens is the same over each 24 h period. Although such study designs are difficult to implement in human trials, they are easily performed in animal and *in vitro* models.

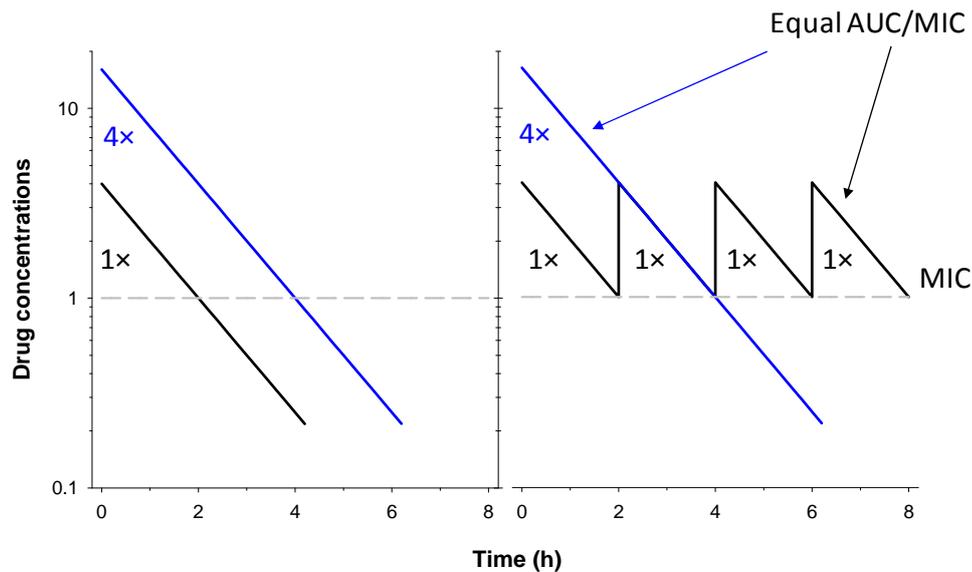


Figure 1-17. Effect of increasing the dose or changing the dosing regimen of a hypothetical drug on C_{\max}/MIC , AUC/MIC and $T_{>\text{MIC}}$. Figure adapted from Craig (376), with permission.

Animal infection studies employing dose-fractionation have linked specific PK/PD parameters with the efficacy of various antibacterial agents against both Gram-positive and Gram-negative organisms (376, 400-401). Additionally, well designed dose fractionation studies allow an estimation of the magnitude of a particular PK/PD index required to be achieved in order to produce a particular level of effect. This is illustrated in Figure 1-18, which shows the relationship between the three PK/PD indices and efficacy of cefotaxime (a cephalosporin) derived from dose fractionation in a murine thigh infection model. As there is good concordance between *in vitro* and animal studies regarding both the PK/PD indices driving antimicrobial

activity and the magnitude of the PK/PD parameters required for efficacy (380, 398), these models will play a crucial role in the rational design of optimal dosage regimens.

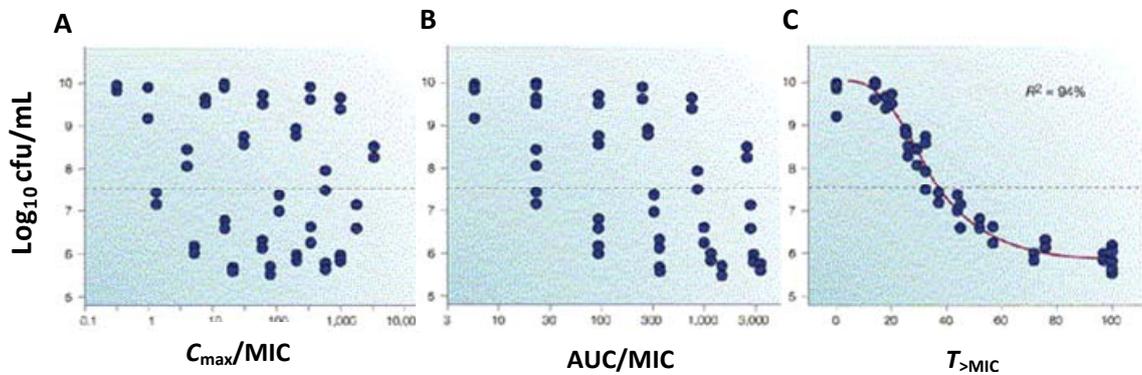


Figure 1-18. The relationship between the three PK/PD indices $C_{\text{max}}/\text{MIC}$ (Panel A), AUC/MIC (Panel B), and $T_{>\text{MIC}}$ (Panel C) and the number of *K. pneumoniae* in the lungs of neutropenic mice after 24 h of therapy with cefotaxime (380). The dashed line represents the number of bacteria at the beginning of therapy. Target exposures ($T_{>\text{MIC}}$) for stasis and near-maximal killing are $\sim 40\%$ and $\sim 65\%$, respectively. Figure adapted from Drusano *et al.* (382), with permission.

PK/PD driver of polymyxin activity: Only recently have studies employed a dose-fractionation design to investigate the relationship between the PK and PD of colistin or polymyxin B, namely which PK/PD index (i.e., the $C_{\text{max}}/\text{MIC}$, the AUC/MIC , or the $T_{>\text{MIC}}$) best correlates with antibacterial activity of colistin (394-395, 402). Tam *et al.* (74) investigated the PD of polymyxin B against 2 strains of *P. aeruginosa* in an *in vitro* PK/PD hollow-fibre model. In that study, an identical daily dose administered 8-, 12-, or 24-hourly did not substantially influence overall bacterial killing, suggesting that the PD of polymyxin B was most closely linked to AUC/MIC . This study, however, was not specifically designed to examine the relationship between efficacy and each PK/PD index. In a conference abstract describing a neutropenic mouse thigh infection model, Keththireddy *et al.* (395) reported that once-daily dosing of colistin was most effective

against *P. aeruginosa*, suggesting that C_{\max}/MIC was the PK/PD index most predictive of efficacy; however, that conclusion could not be confirmed since PK data were not available.

Dudhani *et al.* employed neutropenic mouse thigh and lung infection models in dose-fractionation studies with colistin against three strains each of *P. aeruginosa* (402) and *A. baumannii* (394), including MDR but colistin-susceptible and, for *A. baumannii*, colistin-heteroresistant strains. In these studies the time course of total (i.e., protein-bound plus unbound) and unbound plasma colistin concentrations were determined allowing the PK/PD analysis to be based upon unbound indices (i.e., fC_{\max}/MIC , $f\text{AUC}/\text{MIC}$, and $fT_{>\text{MIC}}$). $f\text{AUC}/\text{MIC}$ was the index most predictive of the antibacterial effect against both *P. aeruginosa* and *A. baumannii* in thigh and lung infection models, indicating that time-averaged exposure to colistin is more important than the achievement of high peak concentrations from the administration of larger, less frequent doses; Figure 1-19 shows the relationship for each index in the thigh model against *P. aeruginosa*. Additionally, the $f\text{AUC}/\text{MIC}$ targets required to achieve various magnitudes of kill (e.g., 2- \log_{10} reduction in viable bacteria) were generally similar across the various strains of the two bacterial species, with some minor differences between infection sites. For example, the $f\text{AUC}/\text{MIC}$ associated with 1- and 2- \log_{10} kill against three *P. aeruginosa* strains in the thigh model ranged from 15.6 to 22.8 and 27.6 to 36.1, respectively (402); the corresponding values against three *A. baumannii* strains were 6.98 to 13.6 and 17.5 to 43.0 (394). In no case was the bacterial burden reduced to below the limit of counting. In the lung infection model, $f\text{AUC}/\text{MIC}$ targets of similar magnitude to those observed in the thigh model were observed for 1- \log_{10} kill in *A. baumannii*, and 1- and 2- \log_{10} kill in *P. aeruginosa*, with somewhat higher values required to achieve greater levels of kill.

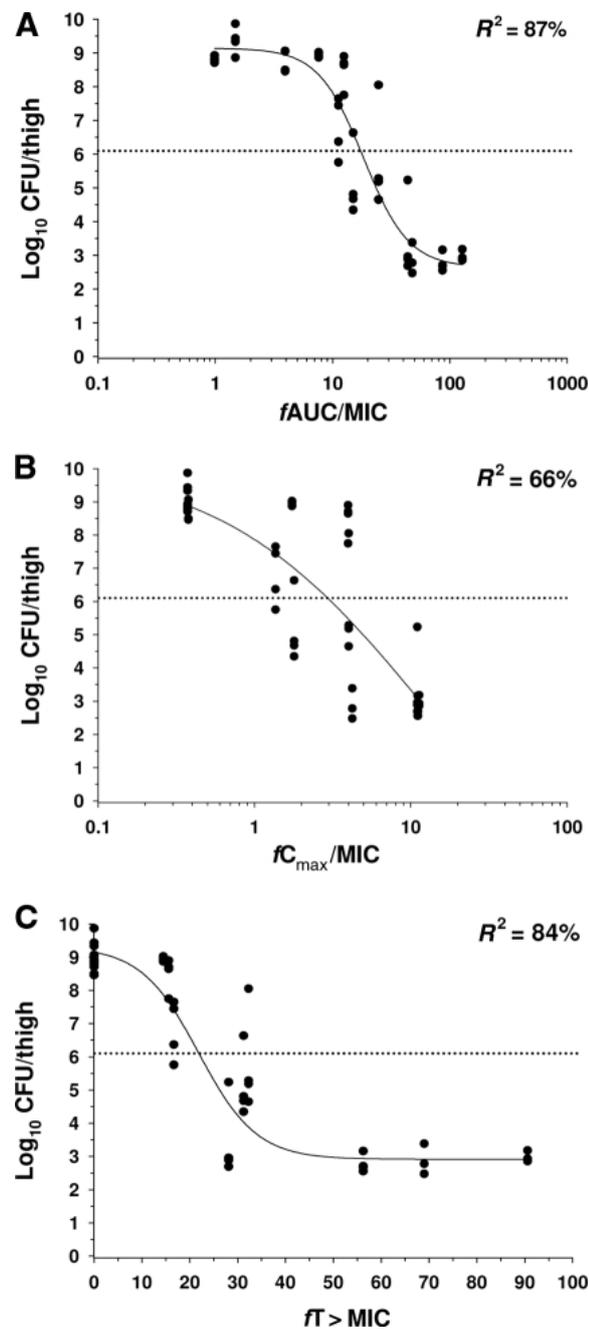


Figure 1-19. Relationships for *P. aeruginosa* ATCC 27853 between the log₁₀ CFU per thigh at 24 h and the PK/PD indices $fAUC/MIC$ (Panel A), fC_{max}/MIC (Panel B), and $fT > MIC$ (Panel C). Each symbol represents the mean datum per mouse from two thighs. R^2 is the coefficient of determination. The dotted line represents the mean bacterial burden in thighs at the start of treatment. Figure reproduced from Dudhani *et al.* (402), with permission.

Unfortunately, as will be discussed in Section 1.5, a lack of information on unbound plasma colistin concentrations means it is currently not possible to compare the $fAUC/MIC$ required for various magnitudes of antibacterial effect observed in the murine models with the $fAUC/MIC$ values for colistin achieved with currently used CMS dosage regimens in patients. Notwithstanding this present difficulty, Garonzik *et al.* (97) utilised the PD data for total colistin (i.e., unbound plus bound) of Dudhani *et al.* (394, 402) and linked them with the average steady-state plasma concentrations ($C_{SS,ave}$; i.e. total plasma concentration) expected to be achieved in individual critically-ill patients from maintenance doses targeting a colistin $C_{SS,ave}$ of 2.5 mg/L. They concluded that in patients with moderate to good renal function it is not possible to achieve colistin $C_{SS,ave}$ values that are likely to be reliably effective without administration of maintenance doses of CMS which may increase the risk of toxicity. This conclusion will be dealt with further in Section 1.6. As further information on PK/PD indices and unbound plasma colistin concentrations is forthcoming it will be possible to assess with greater accuracy not only the ability of current CMS dosage regimens to meet the identified $fAUC/MIC$ targets, but also to design optimised dosage regimens. Chapter 4 of this thesis investigates the PK/PD indices of colistin against *P. aeruginosa* by the conduct of dose fractionation studies in an *in vitro* dynamic model in the absence of protein.

1.5 Pharmacokinetics of CMS and formed colistin

It is only over the last 5 – 10 years that reliable information on the PK of CMS and formed colistin has been forthcoming. Such information has resulted in substantial improvements in our understanding of the disposition of CMS/colistin. The rational design of dosage regimens guided by PK/PD principles will be essential in optimising current therapeutic use to increase the efficacy and prolong the lifespan of this antibiotic.

Colistin is administered parenterally in the form of its sulphomethyl derivative, CMS (Section 1.2.2). Since CMS undergoes hydrolysis to colistin in aqueous media (85-86, 90, 101), including plasma and urine, and as Chapter 2 of this thesis will show that CMS is an inactive prodrug of colistin, it is important to determine the concentrations of both CMS and colistin in order to fully understand the integrated PK of CMS and colistin. Earlier data on the PK (and PD) of CMS and formed colistin in both animals (403-406) and humans (407-409) used microbiological methods for the measurement of drug concentrations; indeed, even some recent studies were undertaken using microbiological assays (410). However, as will be discussed in Chapter 2, microbiological assays are unable to separately quantify CMS and colistin, nor distinguish between colistin A and colistin B, as the assay measures the concentration of active colistin generated from CMS *in vivo* as well as that formed during the incubation period of the assay. Thus, PK analyses utilising microbiological assays to measure 'colistin' concentrations following a dose of CMS most likely represent a complex mixture of CMS, various partially sulphomethylated derivatives, plus colistin. Such assays are incapable of providing accurate information on the time-course of plasma concentrations of either the administered CMS or formed colistin. Furthermore, when samples contain other co-administered antibiotics active against the test strain, the specificity of microbiological assays is further compromised. This is often the case with colistin, which in critically-ill patients is frequently administered concurrently with other antimicrobials active against Gram-negative pathogens. Importantly, the PK and prescribing information supplied with currently available parenteral products was obtained using microbiological assays.

The development of specific analytical methods capable of discriminating between CMS and colistin (base) was a prerequisite for reliable PK studies, with several methods including HPLC with fluorimetric detection following post-column derivatisation (80, 411-412) or, more recently, LC-MS/MS (413-416) now available. The major focus here on reviewing the PK of CMS/colistin

will be on studies conducted following IV administration and employing HPLC or LC-MS/MS analytical methods.

1.5.1 Preclinical studies of colistin and CMS

To understand the complex PK of CMS and colistin it is necessary to first understand the disposition of each. This section examines notable preclinical PK studies; this is important because there are aspects of the overall PK of CMS and formed colistin that are only possible to reveal by undertaking studies involving the separate administration of CMS and preformed colistin, which cannot be readily performed in humans. The emphasis of this section will be on the formation of colistin from administered CMS, the elimination of each, and plasma protein binding.

Li *et al.* (101, 417) was the first to apply a HPLC method capable of distinguishing between CMS and colistin, as well as colistin A and colistin B, to the study of colistin PK. The terminal half-life ($t_{1/2}$) of colistin in rats following a single IV bolus of 1 mg/kg colistin (sulphate) was 74.6 ± 13.2 min (417). The total body clearance (CL) was 5.2 ± 0.4 mL/min/kg, with a renal clearance (CL_R) 0.010 ± 0.008 mL/min/kg; this latter value was far lower than the anticipated clearance by glomerular filtration of 2.3 mL/min/kg, with only $0.18 \pm 0.14\%$ of the total colistin dose recovered in urine. This result indicated very extensive renal tubular reabsorption of colistin and CL mainly via non-renal pathways; a subsequent study in rats confirmed this observation, demonstrating colistin undergoes extensive renal tubular reabsorption via a carrier-mediated process (418). The unbound fraction of colistin (f_u) in plasma in these uninfected animals was relatively concentration independent, with a f_u of ~ 0.44 for total colistin at equilibrium concentrations of 1.5, 3.4 and 6.0 mg/L.

Li *et al.* (101) followed up this initial study with an analogous study in rats administered CMS. Following a single IV bolus of CMS (15 mg/kg), C_{\max} of formed colistin was achieved within 10 min indicating rapid conversion of CMS to colistin. The apparent elimination $t_{1/2}$ of formed colistin was approximately twice that of the administered CMS (55.7 ± 19.3 min versus 23.6 ± 3.9 min), suggesting the elimination of colistin is not rate limited by its formation from CMS, and was similar to the $t_{1/2}$ of colistin administered directly (417). CL of CMS was 11.7 mL/min/kg. In contrast to the low CL_R observed previously for colistin (417), the CL_R of CMS was greater than the glomerular filtration rate (7.2 ± 2.2 mL/min/kg versus ~ 5.2 mL/min/kg, respectively). Even without consideration of plasma protein binding, this result indicated net tubular secretion of CMS into urine. During the first 24 h after dosing $61.1\% \pm 14.4\%$ of the total dose of CMS was recovered in urine, with approximately half present as colistin. However, only $\sim 7\%$ of the administered dose of CMS was converted to colistin systemically. Given this low systemic conversion of CMS to colistin, extensive renal tubular reabsorption of colistin (417), and the fact CMS hydrolyses to colistin in aqueous media including urine (85-86, 90, 101), the high urinary recovery of colistin after administration of CMS was likely an artefact of chemical hydrolysis of CMS at 37°C within renal tubular cells, the bladder and/or at room temperature in the collection vessel. Such a scenario was recently confirmed in healthy volunteers administered a single dose of CMS, with the majority of colistin recovered in urine found to come from renally-excreted CMS (100). A subsequent study in rats by Marchand *et al.* (102) using a wide range of CMS doses (5 – 120 mg/kg IV) confirmed many of the observations of Li *et al.* (101), finding $\sim 16\%$ of the administered dose of CMS was converted to colistin systemically.

Dudhani *et al.* (402) used neutropenic thigh-infected mice and equilibrium dialysis to examine the time course of total (i.e., protein-bound plus unbound) and unbound plasma colistin concentrations following subcutaneous (SC) administration of single doses of colistin sulphate (5 – 40 mg/kg). In contrast to the earlier findings of Li *et al.* (417), colistin plasma protein binding

in these infected animals was concentration dependent across a large plasma colistin concentration range (~1 – 30 mg/L), with the f_u of colistin varying over an ~10-fold range (from ~0.05 – 0.45). As colistin binds not only to albumin but also to the acute-phase plasma protein α_1 -acid glycoprotein (AAG) (419), the increase in f_u with the total plasma colistin concentration likely arises because of the saturation of binding sites as the molar concentration of colistin approaches the molar concentration of AAG. In infected patients, the f_u of colistin in plasma may therefore be expected to be influenced by concentrations of both albumin and AAG, the latter of which may be affected by various pathophysiological stresses including infection (420-421), and potentially to variations in the concentration of colistin (and CMS if it is able to compete with colistin for plasma binding sites [discussed subsequently]). Unfortunately, there is currently no information on unbound plasma colistin concentrations occurring in CMS-treated patients. In addition, the ongoing conversion of CMS to colistin precludes accurate determination of CMS protein binding. Nevertheless, binding of CMS to plasma proteins needs to be considered as competition for binding between CMS and colistin should not be excluded.

1.5.2 Clinical pharmacokinetic studies of CMS and formed colistin

The PK of CMS and formed colistin will be considered separately in patients with CF and critically-ill patients.

1.5.2.1 Studies in patients with cystic fibrosis

As discussed in Section 1.2.5.1, CMS has been used over the last 20 – 30 years administered most commonly by the inhalational route for treatment of *P. aeruginosa* infections in patients with CF (422-428). When administered via inhalation, a small amount of systemic absorption may occur (429-431). Reed *et al.* (99) was the first to examine the PK of CMS/colistin in patients with CF ($n = 31$) receiving IV CMS (from a minimum initial dose of ~13.3 mg CMS/kg/day [~166,000 IU/kg/day] up to ~800 mg CMS/day [10 million IU/day], 8-hourly administration) for

acute pulmonary exacerbation of their disease, reporting 'colistin' C_{\max} values of 21.4 ± 5 mg/L and 23 ± 6 mg/L following the first dose and at steady-state, respectively; the corresponding values for the $t_{1/2}$ of colistin were 3.4 ± 1.4 h and 3.5 ± 1.0 h. However, it was not clear which form was quantified, colistin or CMS, and in addition the HPLC derivatisation method used (which included heating of plasma samples at 54°C or 57°C for 2 h) had the potential to hydrolyse CMS present in plasma at the time of collection to colistin during the analysis process. Thus, the derived parameters are best regarded as apparent values for the fully and partially sulphomethylated derivatives plus colistin.

Li *et al.* (91) performed a similar study in 12 patients with CF using a more reliable HPLC method capable of separately quantifying CMS and formed colistin. Following IV administration of CMS (80 – 160 mg CMS [1 – 2 million IU] 8-hourly), colistin was rapidly formed *in vivo* with substantial formed colistin measurable in all collected samples (the first sample was collected 60 min after the end of the CMS infusion). The steady-state C_{\max} ($C_{\max,ss}$) ranges for CMS and colistin in plasma were 3.6 – 13.2 mg/L and 1.2 – 3.1 mg/L, respectively (Figure 1-20), the latter range considerably lower than that reported by Reed *et al.* (99); the corresponding C_{\min} steady-state ($C_{\min,ss}$) ranges were 0.18 – 2.0 mg/L and 0.14 – 1.3 mg/L. As was observed in rats (Section 1.5.1) (101), the $t_{1/2}$ of formed colistin at steady state was approximately twice that of the administered CMS (4.2 ± 1.3 h versus 2.1 ± 0.87 h); as will be discussed in Section 1.5.2.2, the $t_{1/2}$ of formed colistin in CF patients is considerably shorter than in critically-ill patients. This result is not entirely unexpected as renal function in patients with CF is generally good. As will be discussed in Section 1.5.2.2, renal function significantly affects the apparent CL, and consequently the $t_{1/2}$, of colistin. Recently, similar PK parameters for CMS and formed colistin to those observed in patients with CF were reported in healthy volunteers who received a single dose of 80 mg CMS (1 million IU) (100). In that study, the authors reported that ~30% of the CMS

dose was converted systemically into colistin; however, this is likely to be an overestimate as it fails to account for the possibility of alternate clearance pathways of CMS.

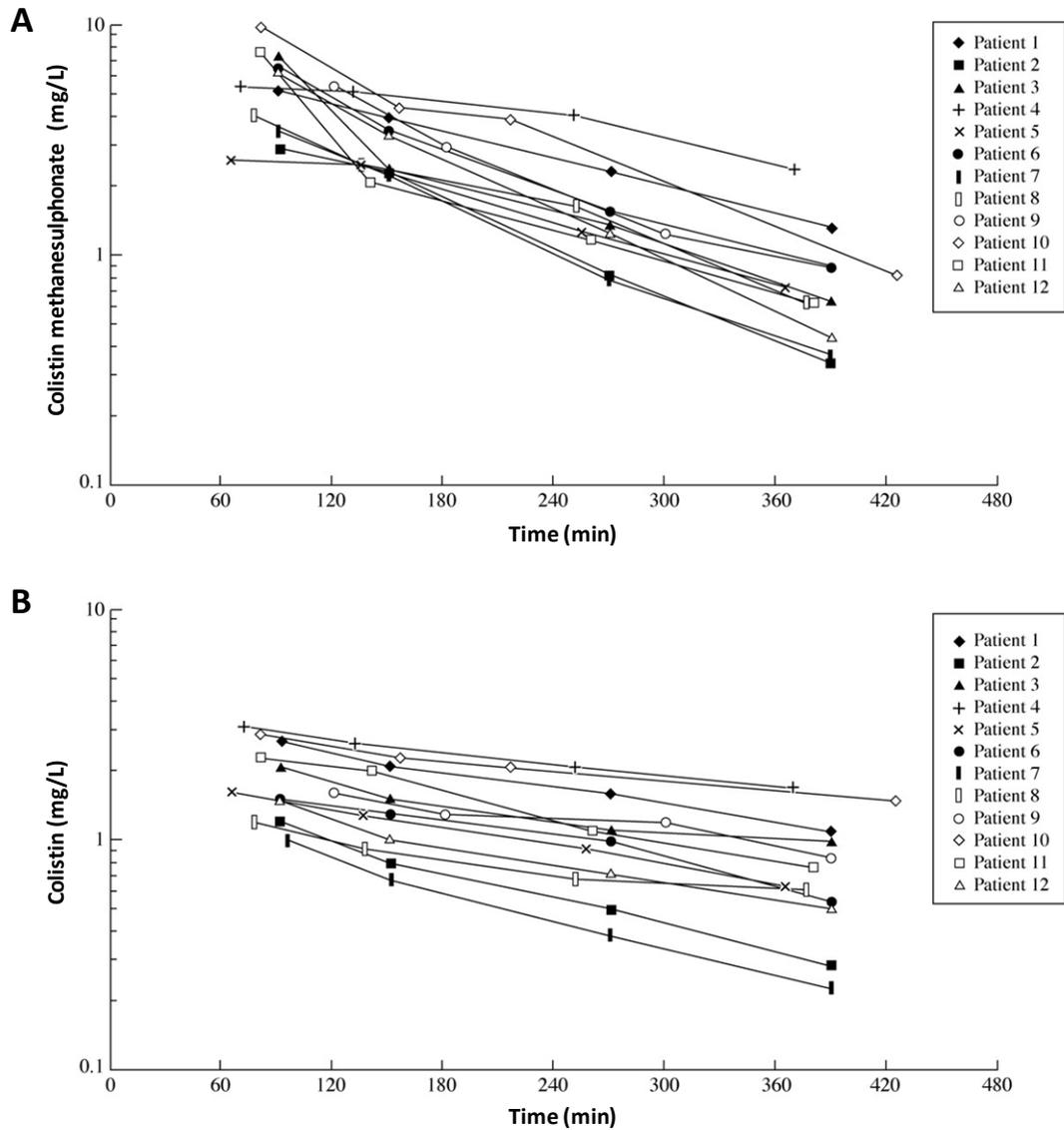


Figure 1-20. Plasma concentrations of colistin methanesulphonate (CMS) (Panel A) and colistin (Panel B) at steady state in 12 patients with cystic fibrosis following IV administration of CMS (80 – 160 mg CMS [1 – 2 million IU] 8-hourly) for at least 2 days. Figure reproduced from Li *et al.* (91), with permission.

1.5.2.2 Pharmacokinetic studies in critically-ill patients

Only recently has the PK of CMS and colistin been investigated in critically-ill patients receiving CMS. Arguably, a 2005 report was the first to draw attention, based upon experimental data, to the lack of PK information for CMS and formed colistin in critically-ill patients and to the lack of appropriate CMS dosage guidelines for these patients (92). That study reported the disposition of CMS and formed colistin at steady state in a critically-ill adult patient requiring CMS for treatment of an infection caused by MDR *P. aeruginosa*. The patient had multiple organ failure requiring continuous venovenous hemodiafiltration (CVVHDF). Neither the product information available in Australia (107) or Europe (105) provides any information on whether CMS (or formed colistin) is cleared by dialysis, whereas the product information available in the USA (106) states that it is not known if CMS or colistin is cleared by dialysis. IV CMS (400 mg CMS/day [5 million IU/day], administered every 24 h) was commenced as last-line therapy; the regimen was based upon the product information which suggested that in patients with renal impairment the size of the dose should be essentially maintained and the dosing interval should be increased from the normal 8 – 12 h. The dose actually administered to this patient was also in accord with the suggestion made, without any supporting data whatsoever, in an influential report focussing upon antibiotic dosing in critically-ill patients receiving continuous renal replacement therapy (CRRT) (432). The C_{max} for CMS was 23.3 mg/L, with the colistin C_{max} (1.84 mg/L) occurring 30 min after completion of the CMS infusion, consistent with the relatively rapid conversion to colistin observed in preclinical studies (101-102) and in patients with CF (91, 99). Importantly, total plasma concentrations of formed colistin fell below the MIC for the infecting strain ~4 h after CMS dosing; the clinical significance of achieved colistin concentrations will be discussed further in Section 1.6. Unfortunately, 12 days after commencing CMS therapy, the patient died. Clearly, dosage adjustment for CMS in CVVHDF patients should be much more modest than that used in this patient.

Imberti *et al.* (93) investigated the steady-state PK and bronchoalveolar lavage (BAL) fluid concentrations of colistin (but not CMS) in 13 adult patients (creatinine clearance [CrCL] 95.5 – 215 mL/min) with VAP treated with IV CMS (174 mg CMS [2 million IU] 8-hourly). Maximum colistin concentrations in plasma occurred ~1 h after the beginning of the infusion, with $C_{\max,SS}$ and $C_{\min,SS}$ values of 2.21 ± 1.08 mg/L and 1.03 ± 0.69 mg/L, respectively. The apparent elimination $t_{1/2}$ and apparent steady-state volume of distribution of formed colistin were 5.9 ± 2.6 h and 1.5 ± 1.1 L/kg, respectively. Although colistin was not detected in BAL fluid (BAL was performed 2 h after the start of the CMS infusion), the authors did not concentrate the BAL fluid prior to analysis to increase the sensitivity of the assay. Therefore, it is not possible to interpret this finding because of the extensive dilution of epithelial lining fluid that occurs during the BAL procedure. Markou *et al.* (94) and Markantonis *et al.* (98) also investigated the PK of colistin (but not CMS) in critically-ill patients. However, with these studies it is unclear whether conversion of CMS (present in samples at the time of collection) to colistin was accounted for when colistin concentrations were determined.

Plachouras *et al.* (95) applied a population PK analysis to 18 critically-ill patients (CrCL 41 – 126 mL/min) receiving IV CMS (240 mg CMS [3 million IU] 8-hourly; 160 mg CMS [2 million IU] 8-hourly if CrCL <50 mL/min). Both colistin and CMS displayed linear PK. CMS concentrations did not accumulate with repeated administration, with a typical C_{\max} value of ~8 mg/L. The predicted colistin C_{\max} values were 0.60 mg/L and 2.3 mg/L following the first dose and at steady-state, respectively. The rate of formation of colistin from CMS in plasma was considerably slower than previously reported in both critically-ill patients and patients with CF (91-93, 96, 101-102), with colistin C_{\max} occurring ~7 h after the start of the CMS infusion and steady-state concentrations achieved after two to three days of treatment. For CMS disposition, a two-compartment model best described the data, with the $t_{1/2}$ values of the two phases for a typical individual being 0.046 and 2.3 h, respectively. The apparent terminal $t_{1/2}$ of colistin, whose disposition was best

described by a one-compartment model, was 14.4 h, considerably longer than previously reported in critically-ill patients (93) and patients with CF (91, 99). For each patient, the profile for formed colistin was much flatter than that for CMS (Figure 1-21). Typical values for the CL of CMS and apparent CL of formed colistin were 13.7 L/h and 9.09 L/h, respectively.

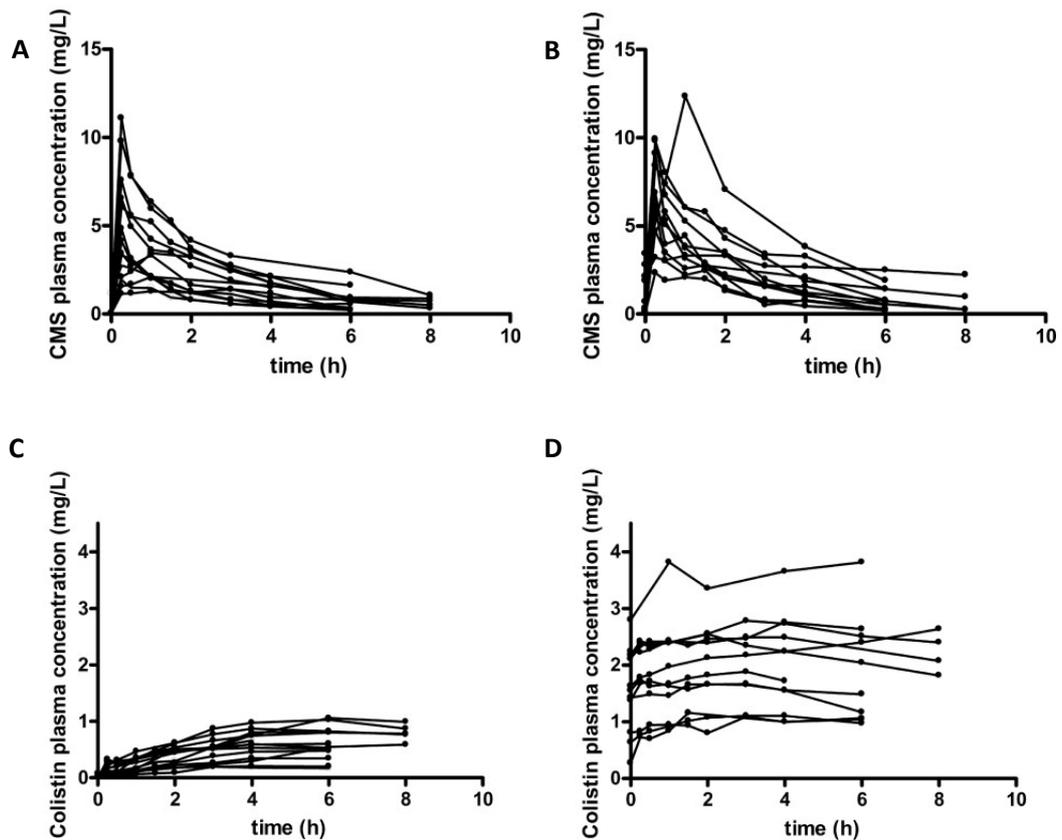


Figure 1-21. Observed individual concentrations of CMS (Panels A and B) and colistin (Panels C and D) in plasma after the administration of the first dose (Panels A and C) and fourth dose (Panels B and D) of CMS. Figure adapted from Plachouras *et al.* (95), with permission.

The PK studies involving critically-ill patients described above contain only a small number of patients, all with relatively good renal function ($\text{CrCl} \geq 41$ mL/min), and with the exception of the case report by Li *et al.* (92), none of the patients was in receipt of renal replacement therapy. Thus, the patient population is unlikely to be representative of the full range of critically-ill

patients who may require CMS. Recently, in what is the largest population PK study examining CMS/colistin yet undertaken, Garonzik *et al.* (97) examined 105 patients (including 12 patients on intermittent hemodialysis [HD] and 4 on CRRT) with very diverse renal function (CrCL 0 – 169 mL/min/1.73 m²; 69 patients had CrCL <40 mL/min/1.73 m²) receiving IV CMS (median daily dose across the 105 patients ~533 mg CMS [6.67 million IU]; range ~200 – 1100 mg CMS [2.5 – 13.67 million IU]) administered 8- to 24-hourly; all but three patients received CMS daily doses within the currently recommended range. As observed by Plachouras *et al.* (95), there was generally little fluctuation in the plasma colistin concentrations across a dosage interval, consistent with a protracted $t_{1/2}$ of formed colistin (Figure 1-22). In those patients not on CRRT, the $t_{1/2}$ of both CMS and formed colistin was dependent on CrCL. Both CMS and colistin were removed efficiently by HD and CRRT, in agreement with previous case reports (92, 433). Substantial inter-patient variability in the plasma concentrations of both CMS and colistin was also observed; the $C_{SS,ave}$ of colistin across all patients ranged from 0.48 – 9.38 mg/L (median 2.36 mg/L).

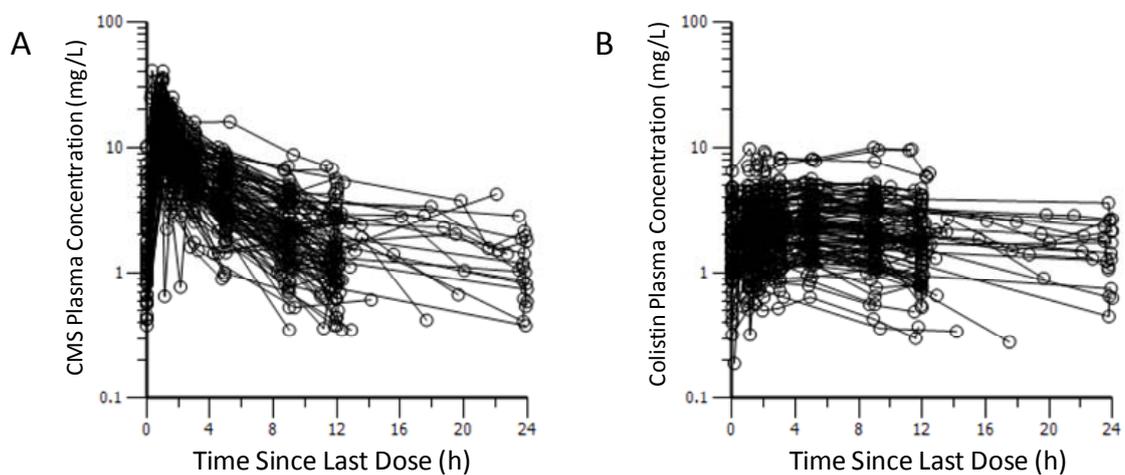


Figure 1-22. Steady-state plasma concentration-time profiles of CMS (Panel A) or formed colistin (Panel B) in 105 critically-ill patients (89 not on renal replacement, 12 on HD and 4 on CRRT). The physician-selected daily dose ranged from ~200 – 1100 mg CMS [2.5 – 13.67 million IU]; the dosage intervals ranged from 8 to 24 h and hence the inter-dosing blood sampling interval spanned the same range. Figure reproduced from Garonzik *et al.* (97), with permission.

The study by Garonzik *et al.* (97) was the first to highlight the importance of renal function on the disposition of CMS and formed colistin. There was a strong inverse trend between the colistin $C_{SS,ave}$ and CrCL (Figure 1-23), and renal function (expressed as CrCL) was an important covariate for the CL of CMS and the apparent CL of colistin. As renal function declined in these patients so too did the CL of CMS, resulting in a greater fraction of the administered dose of CMS converted to colistin (hence the apparent CL of formed colistin was lower in patients with poor renal function). That the CL of CMS correlated with renal function is not surprising given that CMS is predominantly cleared by renal excretion (101). CrCL as a covariate for the apparent CL of colistin, predominantly excreted by non-renal mechanisms (417-418), results from a reduction in CL_R of CMS in patients with substantial reductions in kidney function, allowing a greater fraction of the administered dose of CMS to undergo conversion to colistin; in patients with relatively normal renal function, only a very small fraction of an administered dose of CMS is converted to colistin because the CL_R of CMS is substantially greater than the CL for the formation of colistin from CMS.

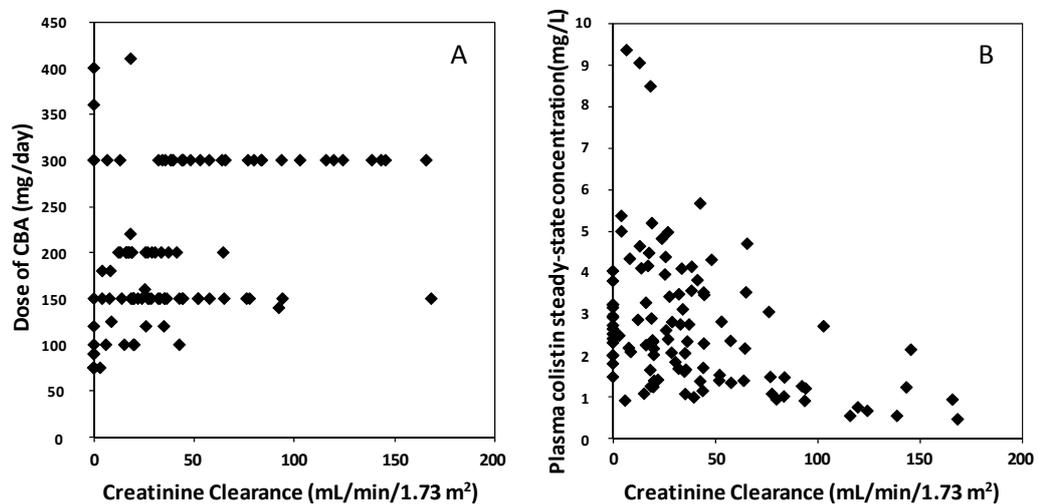


Figure 1-23. Relationship of physician-selected daily dose of colistin (calculated as colistin base activity; CBA) (Panel A) and the resultant average steady-state plasma colistin concentration (Panel B) with creatinine clearance in 105 critically-ill patients. Figure reproduced from Garonzik *et al.* (97), with permission.

Based upon the emerging data on the PK of CMS and formed colistin in animals and humans, the overall disposition of CMS and formed colistin can be summarised as shown in Figure 1-24. It seems very likely that the renal handling of CMS (net secretion, with the possibility of conversion of CMS to colistin within tubular cells (101)) and formed colistin (avid tubular reabsorption (417-418)), processes which serve to traffic CMS/colistin through tubular cells, may be related to the propensity for CMS therapy to cause nephrotoxicity (Section 1.2.5.2).

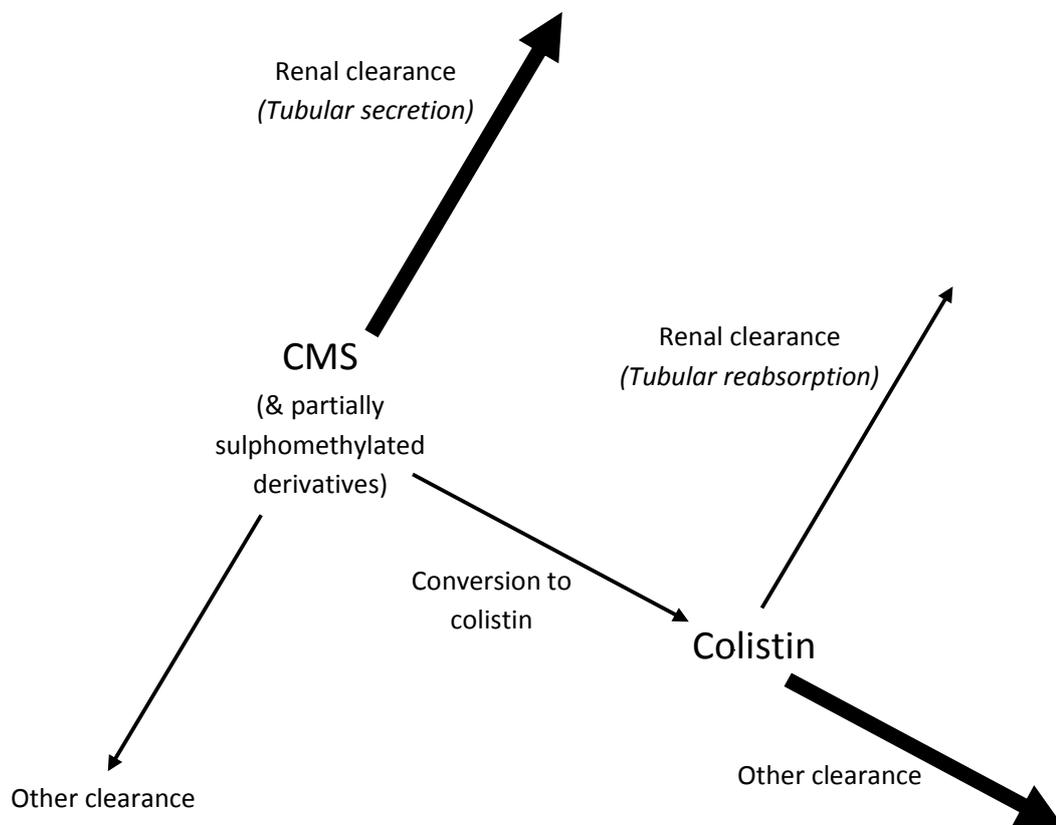


Figure 1-24. Schematic representation of the disposition of CMS and the colistin generated from it in the body following administration of CMS.

Finally, although information on the plasma concentrations of both CMS and formed colistin is increasing, plasma concentrations can only be regarded as surrogate. Unfortunately, information on the disposition of CMS/colistin at infection sites other than plasma following IV

administration of CMS is extremely scarce. As previously discussed, Imberti *et al.* (93) examined penetration of colistin into epithelial lining fluid, but no firm conclusions could be made from that study. Of the remaining extravascular target sites, only penetration into the CNS has been examined in any detail, with negligible permeation of colistin (administered as colistin sulphate) reported across the blood-brain barrier of mice (194-195), and low CMS and colistin penetration into the CSF following IV administration of CMS reported in patients both with, and without, CNS infections (96, 98). Despite this, eradication of Gram-negative pathogens from CSF has been achieved in patients with CNS infections following IV administration of CMS, including patients where poor CNS penetration was observed (96, 162, 189-192). Unfortunately, it is not always possible to ascertain from these reports whether CMS was administered as monotherapy or as part of combination therapy. In addition, publication bias may influence the reported results. As the success of an antibiotic therapy is heavily influenced by the concentration of antibiotic at the site of infection (434-435), further investigations are urgently required to determine the concentrations of CMS/colistin at sites of action other than plasma.

1.6 Appropriateness of current dosage regimens

Several factors have complicated an assessment of the appropriateness of currently administered CMS dosage regimens, not least of which has been until recently a lack of reliable information on the PK and PD of CMS and formed colistin. Current dosage regimens are primarily derived from manufacturers' package inserts developed decades ago and based on PK and PD information derived using microbiological assays. As discussed in Section 1.5, such assays are not suitable for defining the PK and PD of CMS/colistin. Thus, information generated with these assays does not provide a solid scientific basis for understanding the disposition of administered CMS and formed colistin.

In addition to a lack of PK and PD information, substantial variability in dosing recommendations for parenterally administered CMS in renally healthy patients between products used in different parts of the world has caused confusion (Section 1.2.3), potentially leading to a situation of under- or over-dosing. While the added confusion surrounding the optimal dosage frequency and potential for higher, less frequent doses to lead to bacterial regrowth and resistance development has previously been discussed (Section 1.3), *in vitro* studies have additionally shown that the toxic effects of colistin on mammalian cells is both concentration- and time-dependent (436-438), and week-long multiple-dose regimens in rats mimicking once- and twice-daily administration of clinically relevant human daily doses resulted in a greater range and severity of renal lesions with the once-daily dosing equivalent (390). Thus higher doses administered less frequently may potentially increase both nephrotoxicity and resistance development, although this is yet to be confirmed either in patients or, for resistance development, in animal models or *in vitro*. To further complicate this situation, evidence is emerging that the PK of CMS and formed colistin differ across various patient groups (as discussed in Section 1.5), and this may potentially impact the dosage regimens required to treat infections. For example, the $t_{1/2}$ of colistin formed *in vivo* from CMS in CF patients is ~4 h (91, 99), with longer half-lives (up to >14 h (95, 97)) reported in critically-ill patients who may have multiorgan failure, sepsis, or a wider range of renal impairment (92-93, 95, 97); the rate of formation of colistin from CMS may also be substantially lower in critically-ill patients (95).

Despite this confusion, a clearer picture on the appropriateness of currently administered CMS dosage regimens is slowly emerging from recent studies examining the PK of CMS and formed colistin (as outlined in Section 1.5). It is evident from these studies that the colistin C_{max} typically achieved following administration of CMS at the recommended doses are low both in patients with CF (91) and critically-ill patients (92-93, 95, 97). Even without consideration of protein binding, colistin concentrations in many cases either failed to reach the CLSI/EUCAST breakpoint

of 2 mg/L defining susceptibility to colistin for *P. aeruginosa* and *A. baumannii* (152-153), or took considerable time before the breakpoint was reached. For example, the colistin PK profile observed by Plachouras *et al.* (95) in critically-ill patients with doses of CMS 50% greater than recommended by the manufacturer (~240 mg CMS [3 million IU], 8-hourly), although within the dosage range recommended for the US product (106-107), revealed colistin concentrations remain well below the MIC breakpoint for the first few doses. In that study, the predicted colistin C_{\max} was 0.60 mg/L after the first dose, while colistin concentrations ≥ 2 mg/L were not achieved until the sixth dose, a delay of ~44 h; the typical colistin $C_{\max,SS}$ was estimated to be 2.3 mg/L, only marginally above the MIC breakpoint (but without consideration of protein binding). Similarly, Antachopoulos *et al.* (96) observed that in only one of five courses of treatment with CMS did the C_{\max} of formed colistin exceed 2 mg/L. In patients with CF, colistin $C_{\max,SS}$ were below 2 mg/L in a majority of patients, and rapidly fell below this concentration in those patients where it was achieved (91).

Garonzik *et al.* (97) integrated PK data obtained in their study in critically-ill patients with PD data for total (i.e., unbound plus bound) colistin against *P. aeruginosa* (402) and *A. baumannii* (394) from murine thigh and lung infection models (Section 1.4). Assuming the 'average' unbound fraction of colistin in infected humans to be similar to that in mice (417), the authors concluded algorithm-predicted maintenance doses targeting a colistin $C_{ss,avg}$ of 2.5 mg/L would not be reliably effective against isolates with MICs greater than 0.5 mg/L without administering maintenance doses of CMS which may increase the risk of nephrotoxicity. This was especially likely to be the case in patients with moderate to good renal function.

The observations described here are cause for concern. The substantial delay in achieving steady-state colistin concentrations as reported by Plachouras *et al.* (95) places patients at risk as delayed initiation of appropriate antimicrobial therapy is associated with increased mortality

in critically-ill patients (440-441). A loading dose of CMS has been suggested to more rapidly attain steady-state plasma colistin concentrations (95); nevertheless, several hours are still required for achievement of steady-state concentrations of the active antibacterial. Even at steady-state, plasma colistin concentrations achieved with the higher of the two currently recommended dosage regimens (Section 1.2.3) appear suboptimal, particularly against pathogens with MICs in the upper range of the susceptibility breakpoint for colistin (i.e., 2 mg/L) or where infections are associated with high bacterial numbers. Such regimens may result in clinical failures and the development of colistin resistance (126-127, 394). As nephrotoxicity is a dose-limiting adverse effect of CMS in up to ~50% of patients, increasing the daily dose of CMS may not be an option. With resistance to colistin beginning to emerge (39, 45-47, 61-63, 442-443), it is imperative to administer doses that maximise antibacterial activity and minimise resistance development, while also minimising adverse effects (444). Given this situation, combination therapy with CMS/colistin has been suggested as a possible means by which to increase antimicrobial activity and reduce the development of resistance, while minimising potential toxicity (19, 97, 445). Combination therapy will be discussed in Section 1.7 and is investigated in Chapters 5 and 6.

1.7 CMS/colistin in combination with other antibiotics

Although not always successful, combination therapy with antimicrobial agents other than polymyxins has shown favourable results in patients, including against pseudomonal infections (446-449). However, it remains controversial whether combination therapy, given empirically or as definitive treatment for *P. aeruginosa* infection, is warranted (34, 449-451). There are also potential disadvantages with combination therapy including a greater risk of drug toxicity, increased cost, and superinfection with even more resistant bacteria (34). In clinical practice, antimicrobial agents used as part of combination therapy are often selected empirically by clinicians, mainly by trial and error or based on personal experience. This approach is poorly

guided and may not be optimal for patient care. Clearly, quantitative information about increases or decreases in antimicrobial activity (most commonly expressed as synergy or antagonism) and the emergence of resistance is both valuable and necessary for evaluating the effectiveness of antimicrobial combinations.

As outlined in Sections 1.3 and 1.6, the emerging PD and PK data on CMS/colistin suggest that caution is required with monotherapy. Given this situation, CMS combination therapy has been suggested as a possible means by which to increase antimicrobial activity and reduce the development of resistance (19, 97, 445). Two mechanisms have been proposed whereby colistin combinations may provide an enhanced PD effect. As regrowth with colistin monotherapy is due, at least in part, to amplification of pre-existing colistin-resistant subpopulations in heteroresistant strains (126-127, 394), it has been suggested that colistin combinations may give rise to so-called subpopulation synergy, the process whereby one drug kills the resistant subpopulation(s) of the other drug, and *vice versa* (Figure 1-25) (452). Additionally, mechanistic synergy, whereby two drugs acting on different cellular pathways increase the rate or extent of killing of the other drug, has been suggested as a mechanism by which colistin combinations may lead to an enhanced antimicrobial effect (Figure 1-25) (452). The ability of colistin to increase the permeability of the outer membrane of many Gram-negative bacteria (Section 1.2.6) represents one possible mechanism for synergy, potentially allowing better access of other antimicrobial agents to their target sites within the pathogen and thereby improving activity. Mechanisms of subpopulation and mechanistic synergy are not mutually exclusive and both may operate simultaneously.

1.7.1 Clinical studies of CMS combination therapy

At the outset, it should be noted that there are major limitations with all published clinical studies examining CMS in combination with other antibacterial agents due to practical and ethical considerations. Notably, many investigations are retrospective in nature and none have included PK information on CMS, formed colistin, or concomitant antibiotics. In addition, the number of patients participating in existing studies is low and there is heterogeneity in the definitions of outcomes (e.g., mortality or clinical cure), variability in the dosing regimens, and differences in the susceptibility testing methods (disc or broth dilution). Moreover, most studies did not stratify outcome by severity of illness, an important consideration as combination therapy is most likely to be given to the sickest patients who are more likely to die. Finally, there is often no clear rationale for the choice of the second antibiotic. Together, these factors preclude drawing strong conclusions from currently available clinical evidence regarding the benefit or otherwise of CMS combination therapy. As a consequence, clinical studies will be considered here only briefly.

Although CMS is increasingly being used as salvage therapy in combination with one or more antibacterial agents for the treatment of severe infections in critically-ill patients (38, 154, 169), few studies directly compare the effectiveness of CMS monotherapy with combination therapy (67, 160-161, 165, 187, 208, 221, 453-455); these studies, including CMS dosage regimens, are summarised in Appendix 1, Table A1-1. Linden *et al.* (165) reported on 23 critically-ill patients with severe infections caused by *P. aeruginosa* and found no difference in response rates (a favourable response was defined as complete or partial resolution of presenting symptoms and signs by the end of treatment) between patients who received CMS monotherapy ($n = 10$) and patients who received CMS plus either amikacin or an antipseudomonal agent ($n = 13$) (favourable clinical responses of 6 [60%] of 10 vs. 8 [62%] of 13, respectively). Likewise, Falagas *et al.* (221) found no statistically significant difference in clinical response (cure and

improvement) and occurrence of nephrotoxicity between patients with MDR Gram-negative infections receiving CMS monotherapy or CMS/meropenem combination therapy (clinical response 12 [85.7%] of 14 vs. 39 [68.4%] of 57, and nephrotoxicity 0 [0%] of 14 vs. 4 [7%] of 57, respectively). However, in that study a favourable association was revealed between survival and treatment with CMS monotherapy compared to the CMS/meropenem combination (0 [0%] of 14 vs. 21 [36.8%] of 57 deaths), even after adjusting for the variables for which significant differences were found.

In a more recent study by Falagas *et al.* (453) involving 258 patients infected with MDR Gram-negative organisms, infection was cured in an equal proportion of patients (83.3%) who received CMS monotherapy or CMS combined with meropenem; in contrast, patients treated with CMS combined with piperacillin/tazobactam, ampicillin/sulbactam or other agents had significantly lower rates of infection cure (64.7%, 75.0% and 61.3%, respectively). In a subset analysis of patients with infections caused by polymyxin-only-susceptible bacteria (which made up 52.3% of patients in the study), 18 (90%) of 20 patients treated with CMS monotherapy were cured of the infection compared with 70 (83.3%) of 84 patients treated with CMS combined with meropenem and 17 (54.8%) of 31 patients treated with other antimicrobial agents. The multivariate model for cure of infection for this subset of patients showed that treatment with CMS monotherapy or CMS/meropenem combination therapy was an independent factor for cure of infection. In contrast, a much smaller review of studies involving a total of 18 patients with infection caused by KPC β -lactamase-producing *K. pneumoniae* treated with polymyxins (CMS or polymyxin B) alone or in combination reported infections were successfully treated in 1 (14.3%) of 7 patients receiving polymyxin monotherapy and 8 (72.7%) of 11 patients receiving combination therapy (mainly with tigecycline or gentamicin) (456). Finally, in patients receiving antimicrobial therapy for acute respiratory exacerbation of CF, Conway *et al.* (160) found no

substantial differences between patients receiving only CMS ($n = 36$) or CMS plus a second antipseudomonal antibiotic ($n = 35$).

When the results of all studies comparing CMS mono- and combination therapy are considered (Appendix 1, Table A1-1), few significant differences in the effectiveness and safety of mono- and combination therapy are observed, and no clear advantage for CMS combination therapy is evident. However, as noted previously the limitations associated with clinical studies to date significantly hinder interpretation of the data and, as such, the benefits to patients of CMS combination therapy remain unclear. Considering the potential for rapid development of resistance to polymyxins (124) and the unknown clinical implications of heteroresistance, a more systematic examination of the potential for synergy between CMS/colistin and other antibiotics is urgently required. Given the ethical and logistical difficulties involved with comparing CMS monotherapy with combination therapy in patients, preclinical models will play a crucial role in designing and assessing colistin combination therapy.

1.7.2 Preclinical studies of CMS/colistin combination therapy

1.7.2.1 Animal studies

Only a small number of animal studies have examined colistin combination therapy, providing mixed results; these studies are summarised in Appendix 1, Table A1-2. Although many of the factors present in clinical studies which make comparisons of CMS/colistin mono- and combination therapy difficult are absent in animal models (Section 1.7.1), published animal studies to date nonetheless suffer from a number of shortcomings. Unfortunately, it is not always possible to ascertain whether the ‘colistin’ administered in these studies was colistin (sulphate) or CMS (sodium). Although CMS is administered to patients, administration of colistin (sulphate) is preferable in animal models as it permits greater control over the PK profile of the active species, colistin, and facilitates data interpretation; in patients, colistin is formed *in vivo*

following CMS administration (Section 1.5). While it is clear that in several studies CMS was administered (410, 457-458), it is unclear whether the remaining studies administered colistin or CMS (459-462). Importantly, irrespective of the form of 'colistin' utilised, few studies provide a rationale for the doses of CMS/colistin administered. While it appears that the majority of doses utilised have been chosen to reflect human doses on a mg/kg basis, this fails to recognise the importance of animal scaling that results in PK dissimilarities across species (463). Thus, the same or similar mg/kg doses in rodents as administered to humans will achieve substantially lower plasma concentrations in the preclinical species. Crucially, PK data for CMS/colistin and the second antibiotic are absent from virtually all investigations, preventing comparisons with PK profiles achieved in patients; such comparisons are crucial to adequately assess the likely value of the combination in the clinical setting. Given these shortcomings, the results from animal studies reported in the literature (Appendix 1, Table A1-2) are difficult to interpret and considered here only briefly.

Using mouse (460) and rat (459) sepsis models Cirioni *et al.* examined 'colistin' in combination with either imipenem (mouse model) or rifampicin (rat model) against a reference strain and MDR clinical isolate of *P. aeruginosa*; all antibiotics were administered IV. Colistin plus either imipenem or rifampicin resulted in significant reductions in bacterial counts across 72 h when compared with monotherapy with either drug, although only the colistin/imipenem combination resulted in significantly lower mortality. Aoki *et al.* (410) examined the effect of CMS (administered either intranasally or SC) in combination with either imipenem (SC) or rifampicin (orally) against a reference strain and MDR clinical isolate of *P. aeruginosa* using a mouse pneumonia model. Whereas all control mice and mice treated with CMS, imipenem or rifampicin monotherapy ($n = 14 - 16$) died within 42 h of infection with the reference strain, the CMS plus imipenem or rifampicin combinations increased survival to 62.5% and 75% at 72 h, respectively. A clear difference was observed in survival between mice treated with intranasal or SC CMS plus

rifampicin (100% vs. 14%; $P < 0.01$); intranasal CMS was also superior to SC CMS when combined with imipenem. Similar trends were observed using the MDR clinical isolate.

In contrast, two studies found no differences in survival or bacterial clearance from the lungs in mouse pneumonia models with rifampicin monotherapy (administered intraperitoneally (IP); rifampicin was the most active monotherapy) and rifampicin/CMS (IM) combination therapy against MDR *A. baumannii* (457-458). Similarly, there was little difference in survival observed with CMS plus rifampicin against MDR *A. baumannii* in a neutropenic rat thigh infection model (462). However, in this latter study both antibiotics were administered as single IM doses at the beginning of the experiment only. Finally, in the only study of *E. coli*, Giacometti *et al.* (461) examined CMS in combination with piperacillin in a rat intraperitoneal infection model. Following a single IP administration of antibiotics, mortality at 48 h was 93.3%, 33.3%, 33.3%, and 0% for controls, CMS monotherapy, piperacillin monotherapy, and the CMS plus piperacillin combination, respectively ($n = 15$).

Clearly, future animal studies investigating colistin combination therapy which administer colistin (sulphate) and which provide the crucial PK data currently lacking in existing studies are urgently required. Such investigations will be crucial to build on the knowledge gained from *in vitro* studies (discussed in Section 1.7.2.2) and are essential to optimise CMS/colistin therapy.

1.7.2.2 *In vitro* studies

In vitro studies examining combination therapy most commonly define the PD interaction of the agents in terms of additivity, synergy, indifference or antagonism (464). The method used to determine such interactions depends on the experimental system employed. For example, with the checkerboard microbroth dilution method the fractional inhibitory concentration (FIC) index is used. The FIC is calculated as follows (464):

$$\text{FIC index} = \frac{\text{MIC of drug A in combination}}{\text{MIC of drug A alone}} + \frac{\text{MIC of drug B in combination}}{\text{MIC of drug B alone}}$$

With this method, synergy has traditionally been defined as an FIC index of ≤ 0.5 , additivity as an FIC index of 1.0, and antagonism as an FIC index of 2.0 (465-466). However, various definitions are used throughout the literature, and more recent criteria suggest that an FIC index of >4 should be applied to definitions of antagonism to account for inherent imprecision of the technique when twofold dilutions are used and because an FIC index of 2.0 is probably indicative of an indifferent, rather than a true antagonistic, effect (467). Despite its widespread use, the checkerboard method is less discriminatory than other more sophisticated *in vitro* methods (e.g., static or PK/PD time-kill models; discussed below) for assessing the interactions of antimicrobial agents (468-472).

In contrast to the checkerboard technique, which typically provides only inhibitory data, the time-kill method measures the bactericidal activity of the combination being tested. The other major advantage of the time-kill method over the checkerboard technique is that it provides a picture of antimicrobial action over time (based on serial viable counts), whereas the checkerboard technique is usually examined at a single time point (after 16 to 24 h of incubation) (464). Time-kill models can be subdivided into static and PK/PD models. In static time-kill models, with the exception of a small degree of loss in drug activity due to bacterial metabolism or inactivation, bacteria are exposed to static (fixed) concentrations of an antibacterial agent over a defined period of time. In PK/PD models (473-474), the most common of which is the one-compartment model, the test organism is presented with a dynamic concentration of drug designed to mimic *in vivo* PK. PK/PD models (such as that used in Chapters 3, 4 and 6) typically consist of a central reservoir containing the organism, a diluent reservoir and a waste reservoir (Figure 1-26). Drug is added to the central reservoir to achieve the desired peak concentration and the elimination profile is mimicked by addition of sterile, drug-free

media to the central reservoir and removal of an equal volume of drug-containing media into the waste reservoir.

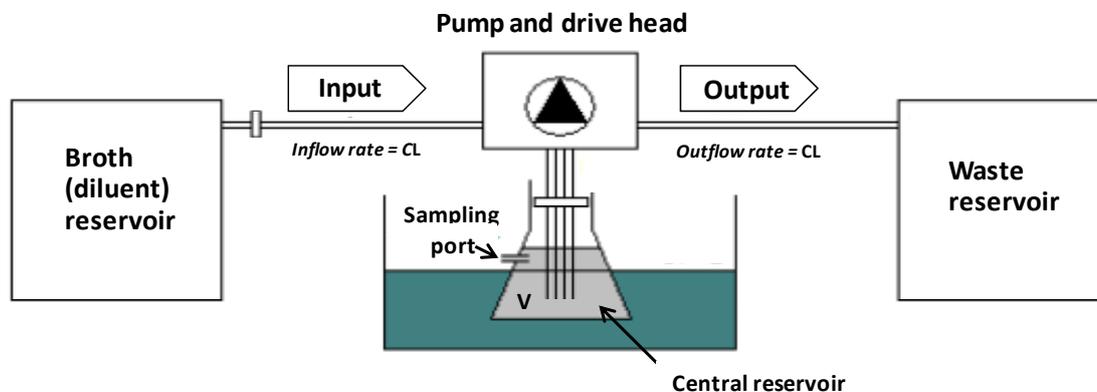


Figure 1-26. Schematic diagram of a one-compartment *in vitro* PK/PD model; CL, clearance; V, volume of distribution.

For both static and PK/PD time-kill methods synergy has traditionally been defined as a 100-fold increase in killing at 24 h (as measured by colony counts; i.e. a $\geq 2\text{-log}_{10}$ lower cfu/mL) with the combination relative to its most active component (Figure 1-27) (464); antagonism is defined as a 100-fold decrease (i.e. a $\geq 2\text{-log}_{10}$ higher cfu/mL) in killing at 24 h with the combination compared with the most active single drug alone. However variations on, and additions to, these definitions abound in the literature, complicating comparisons of effect between studies. For example, synergy is sometimes reported as described above, with the qualification that the number of surviving organisms in the presence of the combination must be $\geq 2\text{-log}_{10}$ cfu/mL below the starting inoculum (475-477). In this way, an interaction described as synergistic by the former definition may not be synergistic by the latter.

Numerous *in vitro* studies have used the static or PK/PD time-kill method to examine CMS/colistin combination therapy. However, the appropriateness of administering CMS in these *in vitro* systems is questionable. As discussed in Sections 1.3 and 1.5 (and demonstrated in Chapter 2), CMS is an inactive prodrug of colistin and undergoes conversion to colistin in

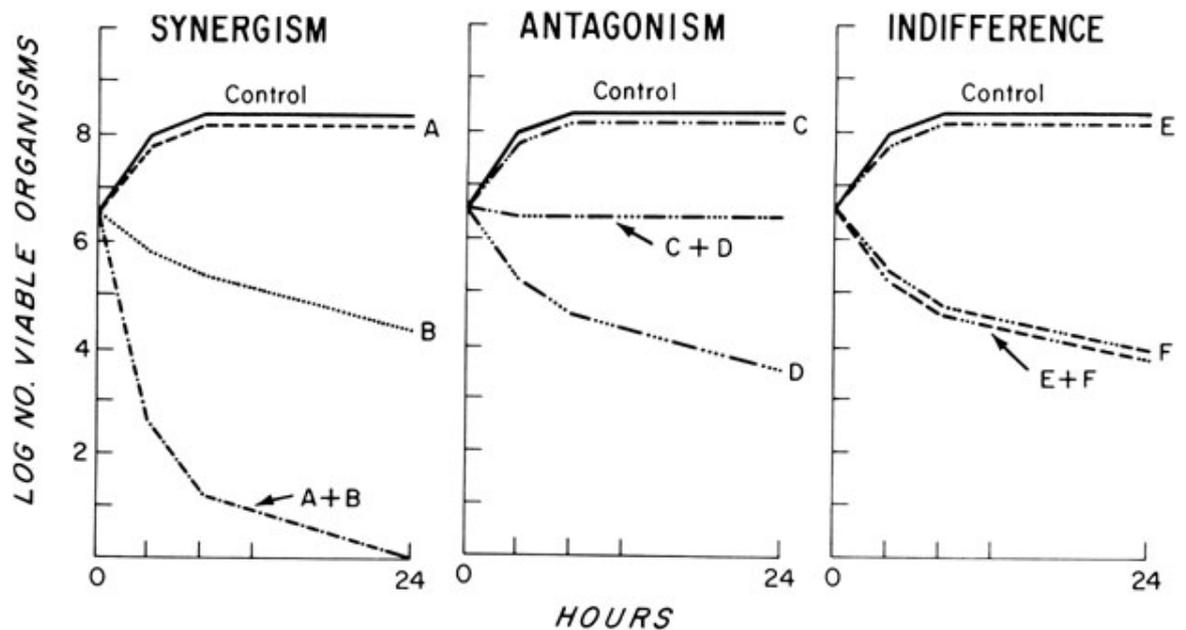


Figure 1-27. Effects of antimicrobial combinations as measured with the time-kill method. A + B, synergism; C + D, antagonism; E + F, indifference. Figure adapted from (464), with permission.

aqueous media (85-86, 90, 101). Administration of CMS will therefore result in a variable formation of active colistin over time. Given this situation, *in vitro* studies which have clearly utilised CMS will not be considered below. Unfortunately, as for animal studies (Section 1.7.2.1) it is not always possible to ascertain whether the 'colistin' administered was colistin (sulphate) or CMS (sodium). Antimicrobial agents combined with colistin in time-kill models include rifampicin (391, 410, 478-480), ampicillin/sulbactam (478), ceftazidime (387), carbapenems (388, 393, 410, 460, 475-476, 478, 481-483), ciprofloxacin (387, 410), aminoglycosides (392, 410, 484), glycopeptides (485-486) and others (482, 487-488); rifampicin and the carbapenems are the most commonly studied antibiotics in combination with colistin. The most common organisms studied are *P. aeruginosa*, *A. baumannii* and *K. pneumoniae*.

Despite the large number of published studies examining colistin combination therapy there are a number of deficiencies with the existing information in addition to the lack of certainty around the form of 'colistin' administered; these deficiencies apply to both static and dynamic (PK/PD) models. The vast majority of studies employ a single inoculum ($\sim 10^5 - 10^6$ cfu/mL). However, as the antibacterial activity of the polymyxins is subject to an inoculum effect (Section 1.3), and as high bacterial densities can be found in some infections, it is important to examine the antibacterial activity of combination therapy at multiple inocula. Consequently, studies incorporating high inocula are warranted to assess the potential of colistin combinations on such infections.

A further deficiency of many studies examining colistin in combination is that the concentrations of antibiotics used, including colistin, are presented as multiples of the MIC with little reference to, or discussion of, the clinical relevance of the actual concentrations used. Further to this, many authors judge the 'success' of a particular combination only by whether synergy was attained rather than examining the overall antimicrobial activity of the combination. Depending on the definition of synergy employed, a combination that attains synergy may still achieve poor overall antimicrobial activity, and may even be less active overall than another combination considered antagonistic. A rigorous analysis of combination therapy which includes regard to clinical applicability of the concentrations employed and the overall antimicrobial activity achieved is warranted.

A major omission with all existing studies examining colistin combination therapy is a consideration of colistin heteroresistance and the effect of combinations on the development of colistin resistance. Colistin heteroresistance is known to contribute to regrowth observed following colistin monotherapy (Section 1.3), although its clinical significance is unclear. However, no existing study of colistin combinations has employed known colistin-

heteroresistant strains. Additionally, no study has examined the emergence of colistin-resistant subpopulations with combination therapy (e.g., via PAPs). Given the status of colistin as an agent of last resort and reports of increasing colistin resistance, it will be crucial to systematically examine the effect of combination therapy on the emergence of colistin resistance, including on heteroresistant strains, in order to design optimal dosage regimens. Finally, remarkably few studies utilise PK/PD models to investigate polymyxins in combination. The introduction of dynamic *in vitro* models has been an important advancement in antimicrobial research, allowing exposure of bacteria to concentrations of antibiotics which change over time according to human PK during treatment (489). Well designed studies in PK/PD systems incorporating the most up to date colistin PK data are urgently required. Significant recent static time-kills studies with colistin (or polymyxin B), as well as all dynamic time-kills studies are discussed below. Studies performed over the last decade are summarised in Appendix 1, Table A1-3; for completeness, studies in which CMS was administered have been included in the appendix.

Static time-kill studies: Souli *et al.* (475) examined colistin (5 mg/L) in combination with imipenem (10 mg/L) against 42 unique clinical isolates of *bla*VIM-1-type metallo- β -lactamase-producing *K. pneumoniae*. After 24 h exposure to the combination, synergy was reported against 12 (50%) of 24 colistin-susceptible isolates, but antagonism was observed against 10 (55.6%) of 18 colistin-resistant isolates. Interestingly, resistance to colistin (MICs 64 – 256 mg/L) was observed in 7 (58.3%) of 12 isolates that were initially susceptible to colistin. In contrast, none of four isolates initially susceptible to imipenem and which showed regrowth at 24 h developed resistance to imipenem. In another study Shields *et al.* (490) examined ‘colistin’ plus doripenem against five MDR isolates of *A. baumannii* taken from patients who had received solid organ transplants; all isolates were susceptible to colistin based on MICs. Against all five isolates doripenem monotherapy at sub-MIC concentrations resulted in virtually no antimicrobial activity, whereas ‘colistin’ monotherapy (0.25 to 1 \times MIC) was bacteriostatic (inhibiting growth of

the inocula without causing significant killing). However, the combination of 'colistin' (0.125 to 0.25× MIC) plus doripenem (8 mg/L) resulted in undetectable bacterial levels at 8 h without evidence of regrowth by 24 h (Figure 1-28). Interestingly, based on these *in vitro* data, combinations of CMS (5 mg/kg/day of CBA in 2 – 4 divided doses) and doripenem (500 mg 8-hourly) were recommended for use in their institution for patients who have received solid organ transplants and were infected with MDR *A. baumannii*. To date, four patients have been treated with this combination with a fifth patient receiving meropenem plus colistin; four (80%) of the 5 patients had a positive clinical response and survived.

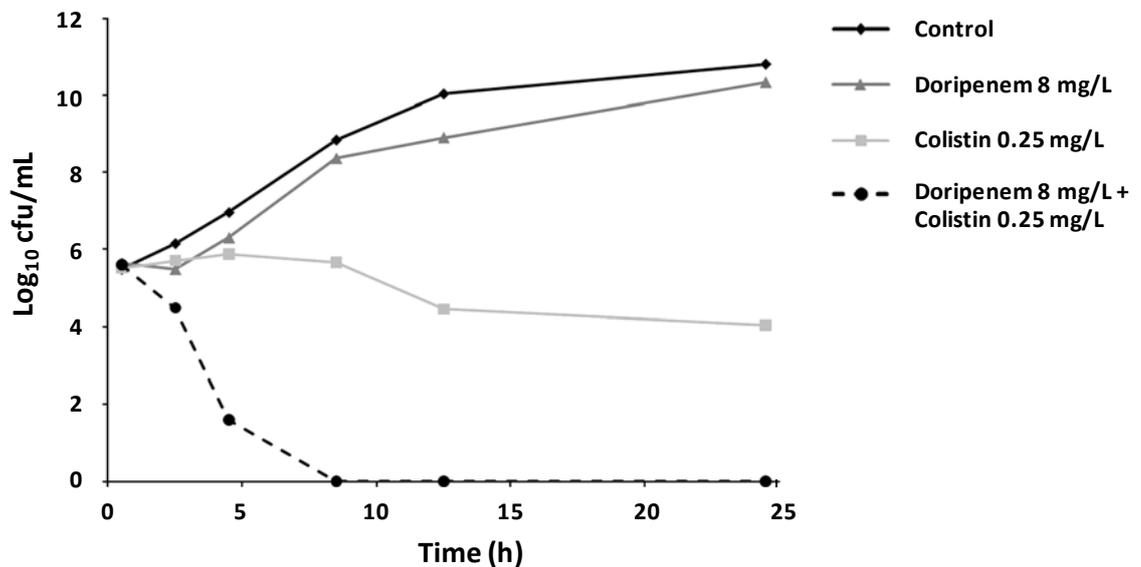


Figure 1-28. Representative time-kill curves with colistin and doripenem alone, and in combination, against a MDR isolate of *A. baumannii*. Figure adapted from Shields *et al.* (490), with permission.

Urban *et al.* (483) examined antibiotic combinations using polymyxin B, doripenem, and rifampicin against MDR isolates of *P. aeruginosa*, *A. baumannii*, *K. pneumoniae* and *E. coli*; all isolates were carbapenem-resistant and all antibiotics were used at a concentration of 0.25× MIC. As monotherapy, none of the tested antibiotics was bactericidal (defined as a ≥ 3 -log₁₀

cfu/mL decrease in 24 h at 0.25× MIC). Triple therapy with the combination of polymyxin B, doripenem and rifampicin was most effective, with bactericidal activity achieved against 5 (100%) of 5 isolates of *P. aeruginosa*, 5 (100%) of 5 isolates of *E. coli*, 4 (80%) of 5 isolates of *K. pneumoniae*, and 3 (60%) of 5 isolates of *A. baumannii*. Combinations utilising only two antibiotics were less effective, with polymyxin B plus doripenem or rifampicin bactericidal against only 1 – 2 (20 – 40%) of 5 isolates for all bacterial species except *E. coli*; against *E. coli*, polymyxin B plus doripenem was bactericidal against 4 (80%) of 5 isolates. In another study, sub-MIC concentrations of colistin (0.12 – 1 mg/L) and meropenem (0.06 – 8 mg/L) were synergistic against 13 (25.5%) of 51 *P. aeruginosa* isolates at 24 h, whereas the combinations of colistin (0.06 – 8 mg/L) and meropenem (0.03 – 64 mg/L) showed synergy against 49 (94.2%) of 52 *A. baumannii* isolates (476). A similar study by the same authors involving colistin (0.12 – 16 mg/L) and doripenem (0.03 – 128 mg/L) demonstrated synergy against 19 (76.0%) of 25 isolates of *P. aeruginosa* at 24 h, whereas colistin (0.12 – 16 mg/L) and doripenem (0.06 – 32 mg/L) showed synergy against 25 (100%) of 25 isolates of *A. baumannii* (481).

PK/PD time-kill studies: Gunderson *et al.* (387) was the first to utilise a one-compartment PK/PD model to examine colistin in combination. In that study colistin (C_{\max} 6 or 18 mg/L, 24-hourly dosing; $t_{1/2}$ 3 h) was combined with either ceftazidime (50 mg/L continuous infusion) or ciprofloxacin (C_{\max} 5 mg/L, 12-hourly dosing; $t_{1/2}$ 3 h) against two colistin-susceptible MDR isolates of *P. aeruginosa*; experiments were conducted over 48 h with an inoculum of $\sim 10^6$ cfu/mL. Although the combination of colistin plus ciprofloxacin generally produced poorer bacterial killing than with either drug alone, the authors reported the combination of colistin plus ceftazidime was synergistic. However, in light of more recent understanding of colistin PK in both critically-ill patients (93, 95, 97) and people with CF (91) (Section 1.5.2), only one maximal concentration (6 mg/L) employed by Gunderson *et al.* (387) can be considered clinically achievable. Additionally, although the simulated 3 h $t_{1/2}$ of colistin is representative of that

observed in patients with CF (91), colistin was administered as a single dose every 24 h. Given colistin is typically administered intermittently to patients every 8 – 12 h, the colistin PK profile generated across a 24-h period was not representative of that observed in CF or critically-ill patients. Moreover, although synergy was defined as a ≥ 2 - \log_{10} decrease in colony count relative to the count obtained with the more active of the two antibiotics alone at 24 h, it appears that only changes in \log_{10} cfu/mL between colistin monotherapy and combination therapy were considered; when data for ceftazidime monotherapy (which was performed for only one of the two isolates tested) is considered, synergy was not observed.

More recently, a small number of conference abstracts have appeared examining colistin in combination with meropenem (393), amikacin (392), and rifampicin (391) against *A. baumannii* utilising PK/PD models. While combinations with meropenem and rifampicin were reported to be synergistic, there are significant limitations with all these investigations, not least of which is that it is unclear whether ‘colistin’ (which was dosed every 12 h) was administered as colistin (sulphate) or CMS (sodium). Additionally, in the two studies where PK data were reported (391, 393), microbiological assays were used to determine ‘colistin’ concentrations. Finally, as for the majority of investigations examining colistin combinations using time-kill methodology, experiments were conducted for 24 h and used a single inoculum ($\sim 5 \times 10^5 - 10^6$ cfu/mL). Given these limitations, while the synergy observed in these dynamic systems is interesting, it is difficult to draw any firm conclusions from these studies.

Clearly, further investigations of colistin combination therapy in static and dynamic PD models incorporating clinically achievable concentrations of colistin and, for dynamic systems, clinically relevant PK profiles are urgently required. Such studies will provide essential information to assist in the design of rational colistin combination therapy. Chapters 5 and 6 of this thesis systematically investigate colistin in combination with carbapenems and utilise both static

(Chapter 5) and dynamic (Chapter 6) time-kill methods. These investigations were undertaken at both low ($\sim 10^6$ cfu/mL) and high ($\sim 10^8$ cfu/mL) inocula, utilised clinically relevant concentrations of each antibiotic, and included colistin-heteroresistant isolates and an examination of the emergence of colistin resistance.

1.8 Summary

The world is facing a growing threat from MDR Gram-negative 'superbugs' such as *P. aeruginosa*, *A. baumannii* and *K. pneumoniae*. This problem is compounded by a lack of novel antimicrobial agents in the drug development pipeline for Gram-negative infections, in particular those caused by *P. aeruginosa*, and novel agents with activity against this pathogen may not be available for some time. This has led to the re-evaluation of colistin (polymyxin E), a once-neglected antibiotic whose use has increased dramatically over the last 10 years, especially in people with cystic fibrosis and critically-ill patients. Colistin still retains significant activity against many MDR Gram-negative bacteria, and is often the only therapeutic option available to treat infections by these pathogens.

Unfortunately, there is a dearth of reliable PK and PD information on colistin and its derivative, CMS (the form administered parenterally), and this has led to confusion regarding the 'optimal' dosing schedule for CMS. With resistance to colistin beginning to emerge, including in *P. aeruginosa*, it is imperative to administer CMS in dosage regimens that maximise antibacterial activity and minimise resistance development and adverse effects. Accordingly, the overarching aim of this thesis was to expand our current knowledge of the PD of colistin and CMS against *P. aeruginosa* in order to optimise use and prolong therapeutic utility. In view of this, the specific aims of this thesis were to:

- 1. Determine the relative contributions to bacterial killing of colistin and CMS against *P. aeruginosa* (Chapter 2):** Static time-kill studies utilising a reference strain of *P. aeruginosa* were performed with CMS; the subsequent formation of colistin from the administration of CMS was measured, generating colistin concentration-time profiles. These colistin concentration-time profiles were subsequently mimicked in other incubations, independent of CMS. The time course of the killing effect between the CMS and colistin-only regimens were then compared to determine the relative contribution of each species to bacterial killing.
- 2. Evaluate the PD of colistin monotherapy against *P. aeruginosa* in terms of antibacterial activity, emergence of resistance, and to identify the PK/PD index most predictive of efficacy (Chapters 3 and 4):** An *in vitro* PK/PD model was used to (i) simulate the PK of formed colistin in humans administered three clinically relevant dosage regimens of CMS, including the currently recommended regimens, and examine the emergence of colistin-resistant subpopulations with each regimen (Chapter 3), and (ii) perform dose fractionation to identify the PK/PD index (i.e., fC_{\max}/MIC , $f\text{AUC}/\text{MIC}$, or $fT_{>\text{MIC}}$) that best predicts colistin efficacy (Chapter 4). Both colistin-susceptible and MDR strains of *P. aeruginosa* were included in the studies.
- 3. Systematically investigate *in vitro* bacterial killing and emergence of colistin resistance with colistin monotherapy and combination therapy against *P. aeruginosa* (Chapters 5 and 6):** Static (Chapter 5) and dynamic (Chapter 6) time-kill studies were conducted at both high and low inocula with colistin and imipenem (Chapter 5) or colistin and doripenem (Chapter 6) alone and in combination. Colistin-susceptible, -resistant, -heteroresistant, and non-heteroresistant strains of *P. aeruginosa* were included in the studies conducted across these two chapters. The emergence of colistin-resistant subpopulations was monitored.

1.9 Structure of this thesis

As the methods employed for the studies contained within this thesis are described in detail in each chapter, a separate methods chapter has not been included. Parts of Chapter 1 (Introduction and Literature Review) formed the basis of a recent review on colistin, which has been accepted for publication (Appendix 3). Chapters 2 to 6, (research findings) comprise manuscripts which have been published or accepted for publication.

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature of my contribution to this manuscript included the following:

- design of the study;
- all laboratory experiments;
- data analysis and interpretation;
- preparation of the initial draft of the manuscript and subsequent revisions; and
- formulation of the conclusions and hypothesis arising from the results of the study.

The extent of my contribution was 65%. The following co-authors contributed to the work:

Name	Nature of contribution
Jian Li	<ul style="list-style-type: none">• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;• review of manuscript drafts and revisions; and• formulation of conclusions and hypotheses arising from the results of the study.
Craig R. Rayner	<ul style="list-style-type: none">• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;• review of manuscript drafts and revisions; and• formulation of conclusions and hypotheses arising from the results of the study.
Roger L. Nation	<ul style="list-style-type: none">• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;• review of manuscript drafts and revisions; and• formulation of conclusions and hypotheses arising from the results of the study.

Candidate's signature

Date

Phillip J. Bergen		
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Chapter Two

Colistin methanesulphonate is an inactive prodrug of colistin against

Pseudomonas aeruginosa

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2.1 Abstract

There is a dearth of information on the pharmacodynamics of 'colistin,' despite its increasing use as a last line of defence for treatment of infections caused by multidrug-resistant Gram-negative organisms. The antimicrobial activities of colistin and colistin methanesulphonate (CMS) were investigated by studying the time-kill kinetics of each against a type culture of *Pseudomonas aeruginosa* in cation-adjusted Mueller-Hinton broth. The appearance of colistin from CMS spiked at 8.0 and 32 mg/liter was measured by high-performance liquid chromatography, which generated colistin concentration-time profiles. These concentration-time profiles were subsequently mimicked in other incubations, independent of CMS, by incrementally spiking colistin. When the cultures were spiked with CMS at either concentration, there was a substantial delay in the onset of the killing effect which was not evident until the concentrations of colistin generated from the hydrolysis of CMS had reached approximately 0.5 to 1 mg/liter (i.e., ~0.5 to 1 times the MIC for colistin). The time-course of the killing effect was similar when colistin was added incrementally to achieve the same colistin concentration-time course observed from the hydrolysis of CMS. Given the killing kinetics of CMS can be accounted for by the appearance of colistin, CMS is an inactive prodrug of colistin against *P. aeruginosa*. This is the first study to demonstrate the formation of colistin in microbiological media containing CMS and to demonstrate that CMS is an inactive prodrug of colistin. These findings have important implications for susceptibility testing involving 'colistin,' in particular, for MIC measurement and for microbiological assays and pharmacokinetic and pharmacodynamic studies.

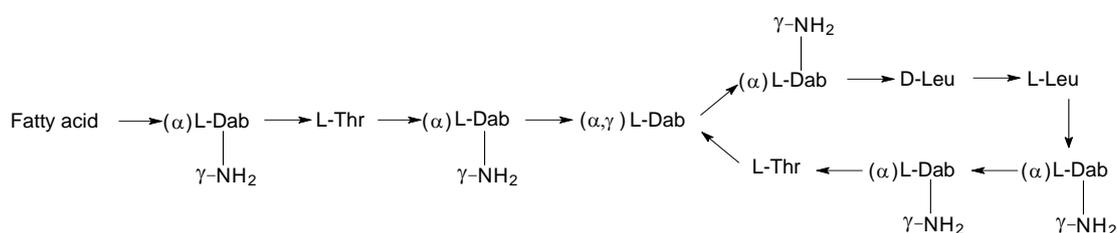
2.2 Introduction

Globally there is a growing threat from the emergence of multidrug-resistant (MDR) microorganisms (9, 57, 491-492). Although the threat from MDR Gram-positive organisms has lessened, at least temporarily, owing to the development of new antimicrobial agents active against these organisms (58), the situation is quite different for MDR Gram-negative bacteria (58-59). With few new antibiotic classes in the drug development pipeline for the treatment of infections caused by MDR Gram-negative bacteria, *Pseudomonas aeruginosa* in particular, we are unlikely to see any new advances in the treatment of infections caused by these organisms in the next few years. Unfortunately, MDR *P. aeruginosa* is increasing in prevalence (25, 57, 493), and infections with this organism are causing major clinical problems in patients with burns, neutropenia, or cystic fibrosis and in those who are immunocompromised (493-495). Several institutions have already experienced outbreaks of *P. aeruginosa* or *Acinetobacter baumannii* infections resistant to all commercially available antibiotics except the polymyxins (171, 496-498). It is precisely this scenario to which the Infectious Disease Society of America refers in its 'Bad Bugs, No Drugs' campaign (9). Given these circumstances, a review of the activity and clinical use of many older antimicrobial drugs is occurring. With their rapid bactericidal activities and current low levels of resistance (40, 111, 245, 499), the polymyxins, and colistin (also known as polymyxin E) in particular, have undergone a revival as agents for treatment of infections caused by MDR *P. aeruginosa*, *A. baumannii* and *Klebsiella pneumoniae* (60, 111, 500). Previously relegated to the status of a reserve agent after early reports of a 'high' incidence of toxicity (54-55), colistin is now a last line of defence for the treatment of infections with MDR Gram-negative organisms (60, 171, 501).

Colistin is a cationic, multi-component lipopeptide consisting of a cyclic heptapeptide with a tripeptide side chain acylated at the N-terminus by a fatty acid (Figure 2-1A). The two major components are colistin A (polymyxin E₁) and colistin B (polymyxin E₂) (75). Two different forms

of colistin available commercially: colistin sulphate (hereafter referred to as colistin) and sodium colistin methanesulphonate (CMS) (Figure 2-1B). CMS is produced by the reaction of colistin with formaldehyde and sodium bisulfite (85), which leads to the addition of a sulphomethyl group to the primary amines of colistin. Colistin is primarily used topically whereas CMS is used parenterally; both forms may be given by inhalation (60). CMS is less toxic than colistin when

A



B

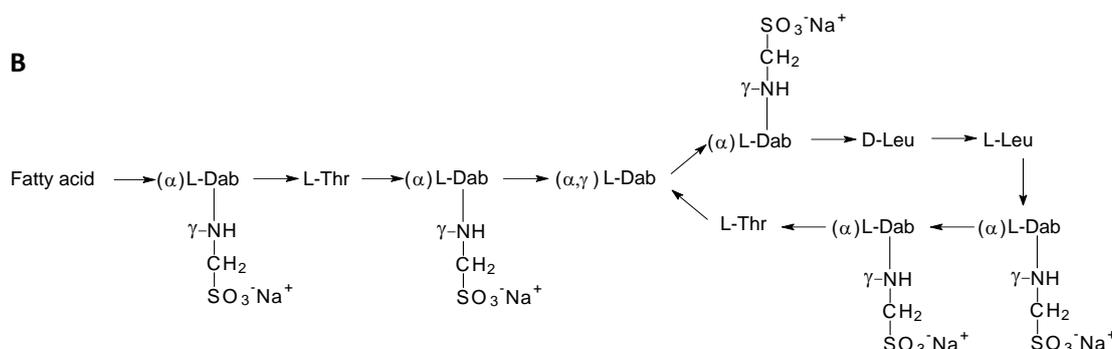


Figure 2-1. Molecular structures of colistin A and B (Panel A); structures of sodium colistin A and B methanesulphonate (CMS) (Panel B). Fatty acid = 6-methyl-octanoic acid for colistin A and 6-methyl-heptanoic acid for colistin B. Thr = threonine; Leu = leucine; Dab = α, γ -diaminobutyric acid. α and γ indicate the respective -NH₂ involved in the peptide linkage.

administered parenterally (86-87), and indeed, this was the reason for the development of CMS. In aqueous solutions CMS undergoes hydrolysis to form a complex mixture of partially sulphomethylated derivatives, as well as colistin (85-86, 90). By the use of high-performance liquid chromatographic (HPLC) methods for separate quantification of CMS and colistin in biological fluids (80, 411, 417), the formation of colistin *in vivo* has been demonstrated in patients (91-92) and rats (101) receiving parenteral CMS; both the CMS administered and the colistin generated circulate in plasma. Furthermore, the pharmacokinetics of CMS and colistin have been demonstrated to differ (91-92, 101).

Even after adjustment for molecular weight differences, CMS has reduced antibacterial activity compared with that of colistin, as assessed by MICs (63, 86-87, 89, 386), and has been reported to be two to four times less active against *P. aeruginosa* (63, 87, 386). However, to our knowledge, no previous publications have reported on the contribution to bacterial killing made by each of CMS and colistin, the latter being formed via hydrolysis from CMS. Knowledge of the relative activities of CMS and colistin has important implications for standardisation of susceptibility studies (e.g., MIC measurement), as well as microbiological assays of 'colistin' in biological fluids; pharmacokinetic and pharmacodynamic studies involving 'colistin' would also be affected. The aim of this study was therefore to determine the relative contribution of colistin and CMS to antibacterial activity against *P. aeruginosa*.

2.3 Materials and methods

2.3.1 Bacterial strains

A reference strain of *P. aeruginosa* ATCC 27853 (American Type Culture Collection, Manassas, VA) was used in this study. The strain was stored at -80°C in a cryovial storage container (Simport Plastics, Quebec, Canada). Fresh isolates were subcultured on horse blood agar (Media Preparation Unit, The University of Melbourne, Parkville, Australia) and incubated at 35°C for 24

h prior to each experiment. Calcium-adjusted Mueller-Hinton broth (CAMHB; lot 332998; Oxoid, Hampshire, England) was used.

2.3.2 Chemicals and reagents

Colistin sulphate (lot 072K1656; 19,530 units/mg) was purchased from Sigma-Aldrich (St. Louis, MO); 200-mg/L and 500-mg/L stock solutions were prepared in water and stored at 4°C before use. Colistin is stable under these conditions (90). Sodium colistin methanesulphonate (lot A1680552; 13,100 units/mg) was purchased from Alpharma Pharmaceuticals (Copenhagen, Denmark); stock solutions of 1,000 mg/L were prepared before each experiment to minimise the potential hydrolysis of CMS in aqueous solutions (90). All stock solutions were filtered by using 0.22- μ m-pore-size Millex-GP filters (Millipore, Bedford, MA). All other chemicals were from the suppliers described previously (80).

2.3.3 Time-kill kinetics

The MIC of colistin (sulphate) and the apparent MIC of CMS for this strain, as determined by broth microdilution (502), were 1 and 4 mg/L, respectively.

2.3.3.1 Colistin methanesulphonate

Time-kill studies were conducted with two concentrations of CMS (8.0 and 32 mg/L) that corresponded to two and eight times the apparent MIC of CMS against *P. aeruginosa* ATCC 27853, respectively. An aliquot (200 μ L) of overnight culture was added to 20 mL of CAMHB and incubated at 37°C until early log-phase growth was reached; 5 mL was then transferred to a 500-mL bottle (Schott Duran, Germany) containing 385 mL of CAMHB, which gave approximately 5×10^5 cfu/mL. CMS was added to achieve an initial concentration of either 8.0 or 32 mg/L. The experiment was conducted for 240 min in a shaking water bath (100 rpm) at 37°C. Serial samples were obtained at 0, 30, 60, 75, 90, 120, 135, 150, 165, 180, 210, and 240 min (2 mL per sample)

for viable cell counting and determination of colistin concentrations and, where relevant, CMS concentrations (see below). Viable cell counting was conducted by spiral plating (WASP2 spiral plater, Don Whitley Scientific Ltd., England) 50 μ L of appropriately diluted sample onto nutrient agar plates (Media Preparation), followed by incubation at 35°C for 18 to 24 h. The colonies were counted with a ProtoCOL colony counter (Don Whitley Scientific Ltd.); the limit of detection was 20 cfu/mL. For each CMS concentration, three replicates were performed, each on a separate day. The time-course of the concentrations of colistin formed by hydrolysis from CMS at each concentration were used as the basis for time-kill experiments involving colistin, as described below.

2.3.3.2 Colistin

The media and inoculum were prepared as described above for CMS. After the inoculation, colistin (sulphate) solution was added at 5-min intervals to mimic the concentration-time course of colistin produced by hydrolysis of CMS at 8.0 or 32 mg/L, determined as described above (corrections were made to allow for differences in molecular weights between the base and the sulphate forms of colistin). Aliquots from either the 200-mg/L or 500-mg/L colistin solutions were used. The experiment was conducted for 240 min in a shaking water bath (100 rpm) at 37°C. Serial samples were obtained at 0, 30, 60, 75, 90, 105, 120, 135, 150, 165, 180, 210, and 240 min (2 mL per sample) for viable cell counting and determination of colistin concentrations. Three replicates were performed at each concentration, each on a separate day. In a control experiment, colistin was added to achieve an initial concentration of 6.0 mg/L (six times the MIC of colistin). Serial samples were obtained at 0, 5, 10, 15, 20, 30, 45, 60, 90, and 120 min for viable cell counting (see above).

2.3.4 Determination of colistin and CMS in CAMHB

Samples from the time-kill studies were taken in duplicate (250 μ L for colistin and 150 μ L for CMS) and placed in 1.5-mL microcentrifuge tubes (Neptune; CLP, Mexico) and immediately stored at -80°C . The concentrations of colistin and CMS were measured by two sensitive HPLC methods previously developed by our group, with minor modifications (80, 411, 417). The assay ranges were 0.083 mg/L to 5.80 mg/L for colistin (base) and 0.25 mg/L to 40 mg/L for CMS. The accuracy and reproducibility of the results for the quality control samples for both assays fell within 10% of target values.

2.3.5 Data analysis

The killing effects from addition of CMS or colistin were examined descriptively and were quantified by calculation of the area under the concentration-time curve of \log_{10} cfu/mL from 0 to 240 min (AUC_{0-240}) normalised by \log_{10} cfu/mL _{$t=0$} , where by \log_{10} cfu/mL _{$t=0$} is the initial \log_{10} cfu/mL value; this was not performed for the control experiment, where colistin was added at 6.0 mg/L at zero time and samples collected for 120 min. AUC_{0-240} was calculated using the linear trapezoidal rule.

2.4 Results

2.4.1 Time-course of colistin formation from CMS

The initial CMS concentrations achieved were 7.64 ± 0.64 mg/L ($n = 3$) for 8.0 mg/L (two times the apparent MIC of CMS) and 29.0 ± 1.98 mg/L ($n = 3$) for 32 mg/L (eight times the apparent MIC of CMS). Figure 2-2A shows the mean colistin concentrations present after the samples were spiked with CMS. The concentrations of colistin present immediately following addition of CMS at 8.0 mg/L were below the limit of quantification of 0.083 mg/L for two of three samples and 0.10 mg/L for the remaining sample; the concentrations present immediately following addition of CMS at 32 mg/L were 0.17 and 0.12 mg/L for two of three samples and below the

limit of quantification for the remaining sample. This indicates that the amount of colistin present in the batch of CMS used was very low. Following addition of CMS, comparatively small amounts of colistin were formed in the first 60 min of incubation: 0.26 ± 0.04 mg/L from 8.0-mg/L CMS and 0.80 ± 0.10 mg/L from 32-mg/L CMS. After 240 min of incubation, these values had risen to 1.49 ± 0.22 mg/L (~1.5 times the MIC of colistin) from 8.0-mg/L CMS and 5.91 ± 0.32 mg/L (~6 times the MIC of colistin) from 32-mg/L CMS; this corresponds (in molar terms) to $29.1\% \pm 2.1\%$ and $30.5\% \pm 2.2\%$, respectively, of the CMS being converted to colistin. The concentration-time profiles of colistin achieved by using incremental spiking of colistin matched closely those derived from the hydrolysis of CMS in CAMHB (Figure 2-2A).

2.4.2 Time-kill kinetics

The time-kill profiles achieved with CMS and colistin at each concentration are shown in Figure 2-2B. In the control experiment in which colistin was spiked at 6.0 mg/L (six times the MIC of colistin), no viable bacteria were detectable after 15 min. When CMS was spiked at 8.0 mg/L, killing began at approximately 90 min when the concentration of colistin formed by hydrolysis of CMS was approximately 0.5× the MIC of colistin (0.46 ± 0.05 mg/L of colistin). When CMS was spiked at 32 mg/L, killing began at approximately 30 min, with a rapid decline in cfu/mL at 60 min, when the concentration of colistin formed by hydrolysis of CMS was approximately 1.0× the MIC of colistin (0.96 ± 0.12 mg/L of colistin). In both cases, very similar time-kill profiles occurred when colistin was added incrementally to achieve the colistin concentration-time courses observed from the hydrolysis of CMS spiked initially with either 8.0 or 32 mg/L CMS. The mean AUC_{0-240} values normalised by initial \log_{10} cfu/mL, $AUC_{0-240}/(\log_{10} \text{ cfu/mL}_{t=0})$, are shown in Table 2-1.

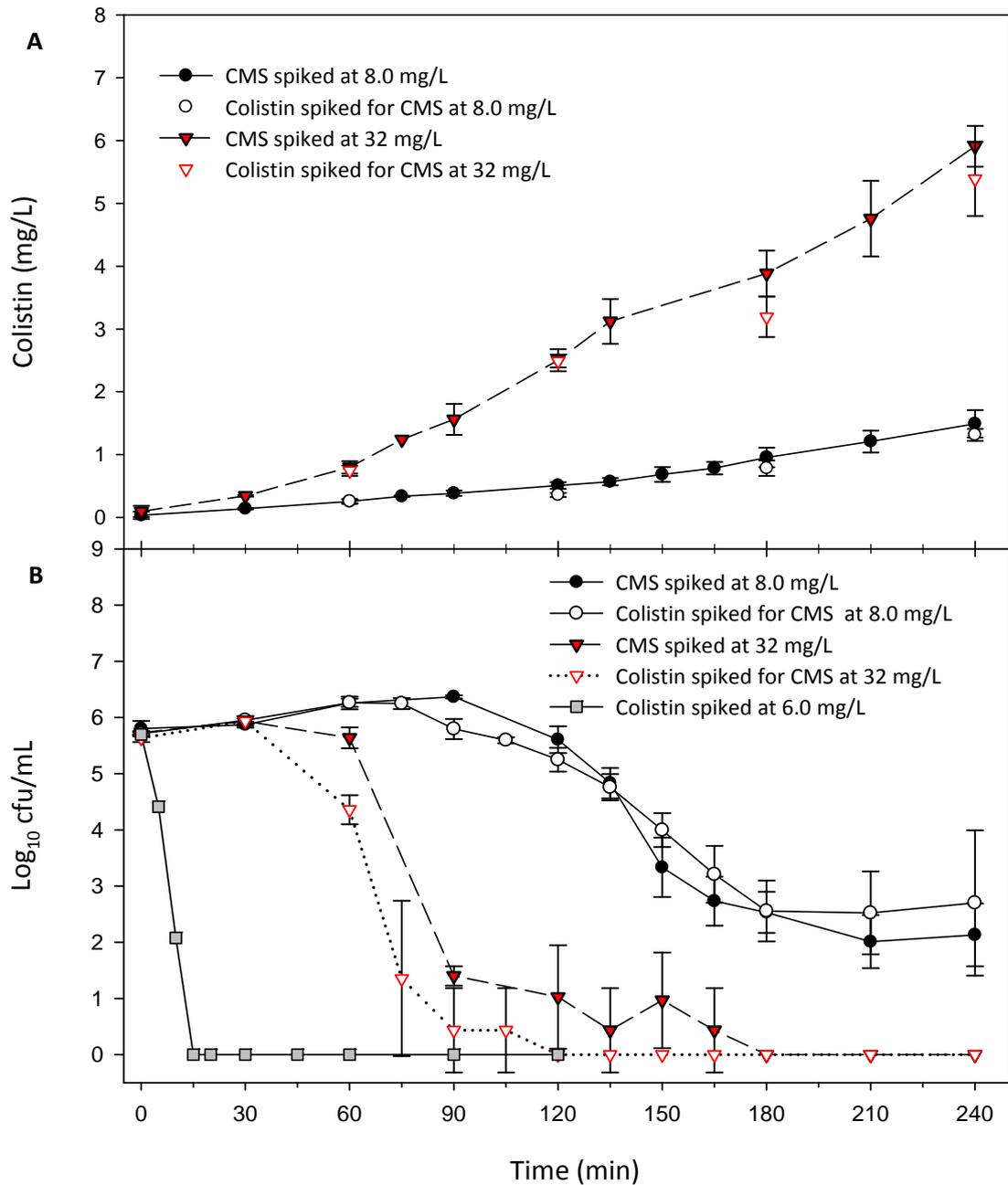


Figure 2-2. (A) Concentration-time course of colistin produced from CMS spiked at 8.0 mg/L and 32 mg/L ($n = 3$) and from incremental spiking with colistin ($n = 3$); (B) time-kill curves for *P. aeruginosa* obtained by using CMS spiked at 8.0 mg/L and 32 mg/L ($n = 3$) at zero time, spiked incrementally with colistin to mimic the colistin concentration-time course achieved after spiking of the sample with CMS at 8.0 mg/L and 32 mg/L ($n = 3$), and colistin spiked at 6.0 mg/L at zero time ($n = 1$; control experiment).

Table 2-1. AUC₀₋₂₄₀ of killing curves normalised by baseline log₁₀ cfu/mL_{t=0}

Colistin form	AUC ₀₋₂₄₀ /(log ₁₀ cfu/mL _{t=0}) (n = 3)	
	8.0 mg/L	32 mg/L
CMS	186.3 ± 6.0	90.4 ± 4.1
Colistin ^a	192.8 ± 10.4	70.0 ± 7.4

^aSpiked incrementally to achieve the same concentration-time course of colistin observed from hydrolysis of CMS spiked initially at 8.0 mg/L or 32 mg/L.

2.5 Discussion

‘Colistin’ is now a last-line of defence against infections caused by MDR Gram-negative organisms (25, 60, 171, 501). Despite renewed interest in the clinical use of ‘colistin,’ confusion has surrounded its use in susceptibility studies, in particular MIC measurement, as well as in microbiological assays used to measure ‘colistin’ concentrations in biological fluids. Information on the pharmacokinetics and pharmacodynamics of CMS and colistin, especially in critically-ill patients, is also lacking (92, 166). Study of the antibacterial activity of CMS, the parenteral form of colistin, has proven complicated due to the hydrolytic conversion of CMS to colistin (90); and this is reflected in the literature (60). Prior to the present study, the relative contributions of CMS and colistin to antibacterial activity have, to our knowledge, never been directly investigated.

The concentrations of CMS chosen for the present study (8.0 and 32 mg/L) represent the concentrations achievable in plasma *in vivo* (91-92). A control experiment in which colistin was spiked at 6.0 mg/L (six times the MIC of colistin), equivalent to the concentration of colistin generated in 240 min after spiking of the samples with CMS at 32 mg/L (Figure 2-2A), demonstrated rapid and extensive killing such that the number of cfu/mL had fallen below the

limit of detection of counting of viable organisms by 15 min (Figure 2-2B); this is consistent with the findings of our previous report (63). In sharp contrast, when CMS was spiked at zero time to achieve concentrations equivalent to two and eight times the apparent MIC for CMS, there was a substantial delay in the onset of the killing effect (Figure 2-2B); in both cases, growth continued for some time after addition of CMS (~75 min for CMS spiked at 8.0 mg/L and ~30 min for CMS spiked at 32 mg/L). This is significant, given that a concentration of CMS even eight times its apparent MIC was unable to cause bacterial killing until significant amounts of colistin had formed. As has already been shown with concentration-dependent anti-infectives, including colistin, bacterial killing occurs at concentrations below the MIC (63, 503). At both CMS concentrations, killing was not evident until the concentrations of colistin generated from the hydrolysis of CMS had reached approximately 0.5 to 1 mg/L (i.e., ~0.5 to 1 times the MIC for colistin).

Importantly, the time-course of the killing effect achieved by spiking with CMS at zero time (8.0 mg/L or 32 mg/L) was very similar to that observed when colistin was added incrementally to achieve the same colistin concentration-time course from the hydrolysis of CMS (Figure 2-2). In particular, the early parts of the killing curves generated from CMS are virtually superimposable on the respective curves from incremental spiking of colistin; this is a time when very little colistin has been generated from CMS. Given that killing did not begin until significant amounts of colistin had formed, as mentioned above, it appears that CMS and the partially sulphomethylated derivatives possess little, if any, antibacterial activity. For each concentration of CMS (8.0 and 32 mg/L), the $AUC_{0-240}/(\log_{10} \text{cfu/mL}_{t=0})$ obtained by spiking of the samples with CMS or spiking of the samples incrementally with colistin to achieve the same colistin concentration-time course (Table 2-1) were within 3.5% and 22.5%, respectively. Consequently, the time-course of antibacterial activity from CMS can be accounted for by the appearance of colistin. Thus, our study has clearly demonstrated that at both concentrations of CMS (8.0 and

32 mg/L), antipseudomonal activity was due to formation of colistin; CMS alone displayed no antibacterial activity. CMS may therefore be regarded as an inactive prodrug of colistin.

The present study has demonstrated that the formation of colistin *in vivo* following administration of CMS is a prerequisite for antibacterial activity. It is also clear that the *in vitro* hydrolysis of CMS to colistin during microbiological procedures conducted in the laboratory (e.g., MIC measurement) has the potential to make CMS appear to possess antibacterial activity. Although we recently reported for the first time colistin formation from CMS *in vitro* (90) and *in vivo* (91-92, 101), to our knowledge no previous work has demonstrated the formation of colistin in microbiological media spiked with CMS during incubation. In the present study, approximately 30% of the CMS present was hydrolysed to colistin after only 240 min. In microbiological procedures such as MIC measurement, where similar temperature conditions but significantly longer incubation periods are used (up to 24 h for MIC measurement), even more colistin would be expected to form. This, together with our demonstration that CMS possesses no (or little) antibacterial activity, has very important implications for microbiological testing procedures.

Due to uncertainties over whether CMS possesses antibacterial activity in its own right, MIC measurements for 'colistin' have been performed using colistin (502) or CMS (111), or both (63, 386). This has caused confusion for clinicians and clinical microbiologists, as evidenced by discussions on the American Society for Microbiology e-mail discussion group (ClinMicroNet, 17 May 2005), with questions such as the relevance of an MIC test performed with colistin for a patient receiving CMS remaining unanswered. The present study has demonstrated that the use of CMS is inappropriate for MIC measurement, as antimicrobial activity is due to the formation of colistin and not to the CMS itself. Recently the Clinical and Laboratory Standards Institute published MIC measurement protocols and stated that colistin, not CMS, should be used when

MICs are determined (502). This decision appears justified by our results. Given the emergence of resistance to colistin (63) and its increasing use, accurate susceptibility data will be vital for the meaningful inclusion of 'colistin' in the testing lists of antimicrobial susceptibility studies; currently, 'colistin' is seldom included in such studies (504).

The demonstration that colistin forms in microbiological media spiked with CMS also has important implications for measurement of 'colistin' concentrations in biological fluids by microbiological assays. Current recommendations for the microbiological assay of 'colistin' involve diffusion methods that use long periods of incubation at elevated temperatures (up to 24 h at 37°C) (505-506), and significant amounts of colistin are likely to form via hydrolysis from CMS under these conditions. After parenteral administration of CMS, *in vivo* hydrolysis ensures that both CMS and colistin will be present at the time of collection of a blood sample (91-92, 101). Over the duration of a microbiological assay, however, significant amounts of colistin will continue to form. Regardless of whether CMS or colistin is chosen as the reference standard against which biological samples from patients are compared, such assays are unable to differentiate between the colistin present at the time of sampling and the colistin formed via the hydrolysis of CMS during incubation. Consequently, microbiological assays of samples collected from patients administered CMS give no information about the individual concentrations of colistin and CMS present at the time of collection.

The inability to accurately determine colistin and CMS concentrations in biological fluids by analytical methods such as microbiological assay, as described above, has significant implications for past and future research on the pharmacokinetics and pharmacodynamics of 'colistin'. Many of the data on the pharmacokinetics of 'colistin' that have been generated were obtained by microbiological assays (160, 407, 507). Some pharmacokinetic studies used HPLC methods that are potentially more specific for measurement of 'colistin' concentrations in humans (99, 508);

however, these particular HPLC methods suffer from problems similar to those encountered when microbiological assays are used. In particular, such HPLC methods (99) cannot differentiate between the colistin present at the time of sampling and the colistin formed by hydrolysis subsequent to sample collection. Thus, most pharmacokinetic data on 'colistin' published to date can be considered representative of a complex mixture of colistin and CMS and its partially sulphomethylated derivatives. Given that colistin formation from CMS occurs *in vivo* (91-92) and, as demonstrated in the present study, that CMS is an inactive prodrug, future studies attempting to define pharmacokinetic and pharmacodynamic parameters must rely on assays that are capable of distinguishing between colistin and CMS, such as the HPLC methods described previously (80, 411).

An explanation as to why CMS displays little, if any, antimicrobial activity may reside in the postulated mechanism of action of the cationic peptides, which includes the polymyxins (245). For Gram-negative microorganisms, it is proposed that the antibacterial activity of polymyxins involves a two-step process that begins with the displacement of divalent cations (Ca^{2+} , Mg^{2+}) on cell surface lipopolysaccharides of the outer membrane, followed by interaction with the negatively charged cytoplasmic membrane (245); the increasing net positive charge of the peptide promotes interaction with each membrane (298-299). Given that at physiological pH the net charge on CMS is -5, the charges on its partially sulphomethylated derivatives range from -3 to +3 (-3 with four attached sulphomethyl groups, +3 with one attached sulphomethyl group), whereas the net charge on colistin is +5, the strongly cationic colistin would be expected to have greater antibacterial activity. While we acknowledge both the limitations of the present study to draw definitive conclusions on this issue and the fact that structural parameters other than charge are important for antibacterial activity in most peptides (298-299, 509-510), the postulated mechanism of action of the cationic peptides combined with the polycationic nature of colistin may explain why CMS acts only as a prodrug.

In conclusion, we have demonstrated the formation of colistin during incubation in microbiological media spiked with CMS by sensitive and specific HPLC methods for the quantification of colistin and CMS. Using a reference strain of *P. aeruginosa*, we investigated the contribution to antimicrobial activity of CMS and its hydrolysis product, colistin, which forms in solution from CMS. Our conclusion that CMS is an inactive prodrug of colistin, as well as our demonstration that colistin forms from CMS in microbiological media, has important implications for susceptibility testing involving 'colistin,' in particular, for MIC measurement, as well as for microbiological assays and pharmacokinetic and pharmacodynamic studies.

2.6 Acknowledgements

The assistance of Roxanne Owen of the Facility for Anti-infective Drug Development and Innovation, Monash University, Melbourne, Australia, is gratefully acknowledged.

This work was supported by Australian National Health and Medical Research Council grant 284265.

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature of my contribution to this manuscript included the following:

- design of the study;
- all laboratory experiments;
- data analysis and interpretation;
- preparation of the initial draft of the manuscript and subsequent revisions; and
- formulation of the conclusions and hypothesis arising from the results of the study.

The extent of my contribution was 70%. The following co-authors contributed to the work:

Name	Nature of contribution
Jian Li	<ul style="list-style-type: none">• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;• review of manuscript drafts and revisions; and• formulation of conclusions and hypotheses arising from the results of the study.
Roger L. Nation	<ul style="list-style-type: none">• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;• review of manuscript drafts and revisions; and• formulation of conclusions and hypotheses arising from the results of the study.
John D. Turnidge	<ul style="list-style-type: none">• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;• review of manuscript drafts and revisions; and• formulation of conclusions and hypotheses arising from the results of the study.
Kingsley Coulthard	<ul style="list-style-type: none">• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;• review of manuscript drafts and revisions; and• formulation of conclusions and hypotheses arising from the results of the study.
Robert W. Milne	<ul style="list-style-type: none">• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;• review of manuscript drafts and revisions; and• formulation of conclusions and hypotheses arising from the results of the study.

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Chapter Three

Comparison of once-, twice- and thrice-daily dosing of colistin on antibacterial effect and emergence of resistance: studies with *Pseudomonas aeruginosa* in an *in vitro* pharmacodynamic model

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3.1 Abstract

Objectives: The optimal dosing regimen for colistin methanesulphonate (CMS) against *Pseudomonas aeruginosa* is unknown. CMS is converted *in vivo* to its active form, colistin. We evaluated three colistin dosage regimens in an *in vitro* pharmacokinetic/pharmacodynamic model.

Methods: Three intermittent dosage regimens involving 8, 12 and 24 h dosage intervals (C_{\max} of 3.0, 4.5 or 9.0 mg/L, respectively) were employed. Antibacterial activity and emergence of resistance were investigated over 72 h using two strains of *P. aeruginosa*: ATCC 27853 and 19056. The areas under the killing curves (AUBC₀₋₇₂) and population analysis profiles (AUCPAP) were used to compare regimens.

Results: No difference in bacterial killing was observed among different regimens. For ATCC 27853, substantial killing was observed after the first dose with less killing after subsequent doses irrespective of regimen; regrowth to between 5.95 and 7.49 log₁₀ cfu/mL occurred by 72 h (growth control 7.46 log₁₀ cfu/mL). AUCPAPs at 72 h for the 12 hourly (4.08 ± 1.54) and 24 hourly (4.16 ± 2.48) regimens were substantially higher than for both the growth control (1.63 ± 0.08) and 8 hourly regimen (2.30 ± 0.87). For 19056, bacterial numbers at 72 h with each regimen (1.32 to 2.75 log₁₀ cfu/mL) were far below that of the growth control (7.79 log₁₀ cfu/mL); AUCPAPs could not be measured effectively due to the substantial killing.

Conclusions: No difference in overall bacterial kill was observed when the recommended maximum daily dose was administered at 8, 12 or 24 h intervals. However, the 8 hourly regimen appeared most effective at minimising emergence of resistance.

3.2 Introduction

The world is facing a growing threat from multidrug-resistant (MDR) microorganisms, especially Gram-negative bacteria (13, 57, 59), and several institutions have already experienced outbreaks of MDR Gram-negative bacteria resistant to all commercially available antibiotics except the polymyxins (60, 171, 496-497, 500). The result has been the increasing use of colistin (also known as polymyxin E) as an agent of last resort for treating infections caused by MDR Gram-negative organisms (38-39, 171, 501). However, knowledge of the pharmacokinetics (PK) and pharmacodynamics (PD) of colistin is limited, and resistance to the polymyxins has recently emerged (494, 511-514). With few new therapeutic options becoming available in the foreseeable future, particularly for *P. aeruginosa* (13), solid PK/PD data on colistin is urgently needed. Such information will be crucial in determining optimal dosing strategies to maximise the clinical benefit of, minimise the development of resistance to, and prolong the usefulness of this increasingly important therapeutic option.

Colistin is available commercially as colistin sulphate (hereafter referred to as colistin) and sodium colistin methanesulphonate (CMS). Owing to reduced toxicity when compared with colistin (86-87), CMS is used parenterally whereas colistin is primarily used topically. The formation of colistin *in vivo* following parenteral administration of CMS has been demonstrated in both rats (101) and humans (91-92). Recently, we established that CMS is an inactive prodrug of colistin (515).

Due to the limited knowledge of the PK and PD of colistin and CMS, confusion surrounds the optimal dosing regimen that maximises antibacterial activity and minimises the emergence of resistance (39, 60). At present 8 (160, 162, 167, 500), 12 (162) and 24 hourly (387, 389) dosage regimens of CMS are all used clinically in patients with normal renal function. The aim of this study was to evaluate the PD of colistin against *P. aeruginosa* in terms of antibacterial activity

and emergence of resistance. This was achieved by simulating, in an *in vitro* PK/PD model, the PK of colistin formation in humans administered three clinically relevant dosage regimens of CMS, including the currently recommended regimens (106-107). Given that the antibacterial activity of CMS results from its hydrolysis to colistin (515), the PK/PD parameters used to describe the activity of 'colistin' must be based on the concentrations of colistin present, not CMS. The PK parameters (C_{\max} and $t_{1/2}$) used in our studies were based on reliable clinical PK data for colistin (63). This study was not designed to determine the optimal PK/PD index for colistin.

3.3 Materials and Methods

3.3.1 Bacterial strains and media

Two strains of *P. aeruginosa* were employed in this study: a reference strain, ATCC 27853 (American Type Culture Collection, Rockville, MD, USA) and a clinical isolate, 19056 (mucoid) from a patient with cystic fibrosis. The MICs of colistin (sulphate), as determined by broth microdilution (502), were 1 mg/L for ATCC 27853 and 0.5 mg/L for 19056; both strains were stored in tryptone soy broth (Oxoid Australia, West Heidelberg, Victoria, Australia) with 20% glycerol (Ajax Finechem, Seven Hills, NSW, Australia) at -80°C in cryovial storage containers (Simport Plastics, Boloelil, Quebec, Canada). Prior to each experiment, strains were subcultured onto horse blood agar (Media Preparation Unit, The University of Melbourne, Parkville, Australia) and incubated at 35°C for 24 h. One colony was then selected and grown overnight in 10 mL of cation-adjusted Mueller-Hinton broth (CAMHB; Oxoid, Hampshire, England) from which early log-phase growth was obtained.

3.3.2 Chemicals and reagents

Colistin sulphate (lot 095K1048; 20,195 units/mg) was purchased from Sigma-Aldrich (St Louis, MO). Immediately prior to each experiment, colistin stock solutions were prepared using Milli-Q water (Millipore Australia, North Ryde, NSW, Australia), sterilised by a 0.22- μm -pore-size Millex-

GP filter (Millipore, Bedford, MA), and then stored at 4°C before use; colistin is stable under these conditions (90). All other chemicals were from suppliers previously described (80).

3.3.3 *In vitro* PK/PD model

The studies examined the effect of three different colistin dosing regimens (discussed subsequently) on microbiological response and emergence of resistance and were conducted over 72 h using a one-compartment *in vitro* PK/PD model. Briefly, the system consisted of four sealed reservoirs (compartments) each containing 100 mL of CAMHB (Ca^{2+} 23.0 mg/L, Mg^{2+} 12.2 mg/L) and a magnetic stir bar to ensure adequate mixing. Each experiment was conducted using three replicates, with the remaining (drug-free) reservoir acting as a control to define growth dynamics in the absence of colistin. All reservoirs were heated in paraffin oil to 37°C throughout the experiment. A peristaltic pump (Masterflex[®] L/S[®], Cole-Parmer, USA) was used to deliver sterile (drug-free) CAMHB from a separate sealed reservoir into each of the four compartments at a predetermined rate (0.3 mL/min), displacing an equal volume of CAMHB into a waste receptacle. This produced a $t_{1/2}$ of 4 h for colistin administered into the central reservoirs; this approximates the $t_{1/2}$ determined in cystic fibrosis patients with normal renal function (91). At the beginning of each experiment, a 1.0 mL aliquot of early log-phase bacterial suspension, obtained from overnight culture, was inoculated into each reservoir giving $\sim 10^6$ cfu/mL. Colistin was administered to each treatment reservoir to achieve the desired C_{max} as described below and in Table 3-1. Serial samples (1 mL) were collected aseptically from each reservoir via a rubber septum-sealed port for viable cell counting and population analysis profiles (PAPs), as well as determination of colistin concentrations (discussed subsequently).

Three intermittent colistin dosage regimens were simulated (Table 3-1). At the beginning of each experiment, the appropriate loading dose of colistin (sulphate) was injected into three of the four reservoirs followed by intermittent maintenance doses at 8, 12 or 24 h intervals. The

8 hourly dosage regimen closely simulated the expected plasma unbound peak ($C_{\max} = 3$ mg/L) (91) and trough ($C_{\min} = 0.75$ mg/L) concentrations of colistin at steady state when CMS is administered 8 hourly according to the manufacturer's recommendations (5 mg/kg/day of colistin base activity; Coly-Mycin™ M Parenteral package insert [Monarch Pharmaceuticals, Bristol, TN, USA]) in patients with normal renal function. The 12 and 24 hourly dosage regimens were designed to achieve higher C_{\max} values (Table 3-1) with extended dosage intervals.

Table 3-1. Colistin (sulphate) dosage regimens, PK/PD indices, and sampling times in the *in vitro* PK/PD model

	8 hourly dosing	12 hourly dosing	24 hourly dosing
Loading dose (mg)	0.30	0.45	0.90
Maintenance dose (mg)	0.23	0.39	0.89
Target C_{\max}/C_{\min} (mg/L)	3.0/0.75	4.5/0.56	9.0/0.14
ATCC 27853/clinical isolate 19056			
AUC/MIC ^a	39.0/77.9	45.4/91.0	51.1/102.3
C_{\max}/MIC^a	3.0/6.0	4.5/9.0	9.0/18.0
$T_{>MIC}^a$	79.3/100	72.3/100	52.8/69.5
Sampling times (h) for microbiological measurements ^b	0, 1, 2, 4, 6, 8, 9, 16, 17, 24, 25, 26, 28, 30, 32, 33, 40, 41, 48, 49, 50, 52, 54, 56, 57, 64, 65 and 72	0, 1, 2, 4, 6, 8, 12, 13, 24, 25, 26, 28, 30, 32, 36, 37, 48, 49, 50, 52, 54, 56, 60, 61 and 72	0, 1, 2, 4, 6, 8, 24, 25, 26, 28, 30, 32, 48, 49, 50, 52, 54, 56 and 72

^a Target values of PK/PD indices.

^b cfu/mL determined for all samples. PAPs were performed at times 0, 24, 48 and 72 h only.

3.3.4 Microbiological response and the emergence of resistance to colistin

Sampling times are shown in Table 3-1. Viable counting and PAPs were conducted immediately after sampling by spiral plating (WASP2 spiral plater, Don Whitley Scientific Ltd., England) 50 μ L of appropriately diluted sample (using 0.9% saline) onto either nutrient agar (viable counting in *in vitro* PK/PD model) or Mueller-Hinton agar (PAPs), followed by incubation at 35°C for 24 h.

PAP 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 (91). Enumeration was performed using a ProtoCOL colony counter (Don Whitley Scientific Ltd.); the limit of detection was 20 cfu/mL.

3.3.5 Determination of colistin concentration in CAMHB

Samples (250 μ L) collected from the *in vitro* PK/PD experiments were placed in 1.5-mL microcentrifuge tubes (Neptune™, CLP, Mexico) and immediately stored at -80°C until analysis. Concentrations of colistin were measured using HPLC (80, 417). The assay range for colistin was 0.10 mg/L – 6.00 mg/L; samples were diluted when the expected colistin concentrations were higher than the upper limit of quantification. Analysis of quality control samples with nominal concentrations of 0.40 and 4.00 mg/L had measured concentrations of 0.34 ± 0.03 mg/L ($n = 26$) and 4.27 ± 0.29 mg/L ($n = 26$), respectively; a quality control sample with nominal concentration of 9.00 mg/L was used to assess the accuracy and reproducibility of the dilution step and had a measured concentration of 9.51 ± 0.13 mg/L ($n = 6$).

3.3.6 Data analysis

Microbiological response to each regimen was examined graphically and quantified by calculation of the area under the killing curve of \log_{10} cfu/mL from 0 to 72 h (AUBC₀₋₇₂); this area was normalised by dividing by the initial inoculum (i.e. \log_{10} cfu/mL at time zero). Changes in the PAPs for ATCC 27853 were examined descriptively and quantified by calculating the area under the PAPs curve (AUCPAP) normalised by the respective PAP inoculum. AUBC₀₋₇₂ and AUCPAP were calculated using the linear trapezoidal rule. Unless otherwise indicated, data are expressed as mean \pm SD.

3.4 Results

3.4.1 Colistin concentrations achieved for each simulated dosage regimen

For the 8, 12 and 24 hourly dosage regimens (Table 3-1), the mean measured concentrations immediately after dosing were 3.44 ± 0.38 ($n = 6$), 4.63 ± 0.34 ($n = 6$), and 9.30 ± 1.58 ($n = 6$) mg/L for the targeted C_{\max} values of 3.0, 4.5 and 9.0 mg/L, respectively. The mean colistin $t_{1/2}$ across all experiments determined from the measured concentrations was 4.13 ± 0.49 h ($n = 18$) for the targeted value of 4 h.

3.4.2 Microbiological response

The time-course profiles of bacterial numbers achieved with all dosage regimens for each strain are shown in Figure 3-1. Substantial differences in total killing were observed between the two strains, with the clinical isolate 19056 exhibiting greater kill than ATCC 27853. All dosing regimens for both strains resulted in extensive bacterial killing to the limit of detection ($>5 \log_{10}$ reduction in cfu/mL) within 1 – 2 h of the first administration of colistin.

For ATCC 27853, regrowth after the initial administration of colistin occurred within 6 h with all regimens (Figure 3-1A), despite the colistin concentrations at this time (~ 1.0 , 1.6 and 3.2 mg/L for the 8, 12 and 24 hourly dosage regimens, respectively) remaining at or above the MIC of 1 mg/L. Although bacterial numbers declined after each subsequent administration of colistin, the extent of the decrease was less and generally never attained the previously undetectable levels observed following the first colistin dose. In addition, following the small decrease in bacterial numbers, regrowth occurred after each dose, as was observed after the first dose. At 72 h, bacterial numbers for the 24 hourly dosage regimen were virtually superimposable with those for the growth control, while those for the 8 and 12 hourly regimens were ~ 1.5 and $\sim 0.7 \log_{10}$ cfu/mL below the control, respectively (Figure 3-1A). The general similarities in time-courses for

bacterial response to each regimen are reflected in small differences in AUBC₀₋₇₂ values (Table 3-2).

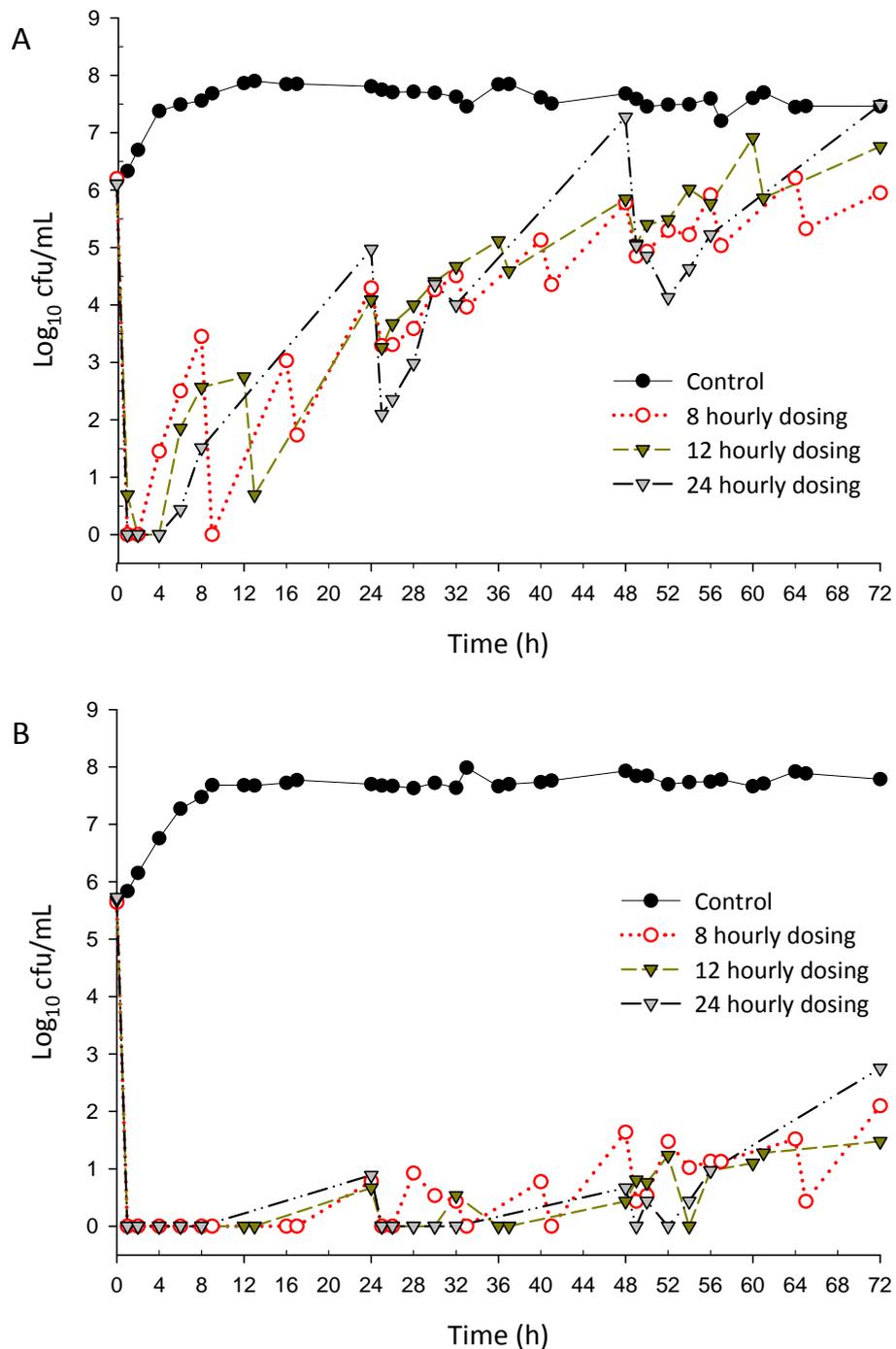


Figure 3-1. Microbiological response observed in the *in vitro* PK/PD model simulating the colistin pharmacokinetics ($t_{1/2}$ of 4 h) of different dosage regimens: 8 hourly dosing (C_{\max} 3 mg/L), 12 hourly dosing (C_{\max} 4.5 mg/L) and 24 hourly dosing (C_{\max} 9 mg/L) for (A) ATCC 27853 and (B) clinical isolate 19056. Data are presented as mean values.

Table 3-2. AUBC₀₋₇₂ of the time-course of microbiological response (normalised by initial inoculum) in the *in vitro* PK/PD model

Strain	AUBC ₀₋₇₂			
	control	8 hourly dosing	12 hourly dosing	24 hourly dosing
ATCC 27853	88.4 ± 1.18	46.6 ± 1.30	50.4 ± 4.56	51.6 ± 5.75
19056	96.5 ± 0.81	8.30 ± 8.74	6.53 ± 5.77	8.43 ± 2.70

Compared to ATCC 27853, regrowth for clinical isolate 19056 occurred more slowly after initiation of all colistin regimens, and to a much lower extent (Figure 3-1B). It was not possible to detect regrowth with any of the regimens until 24 h. Thereafter, regrowth of bacteria was detected with each regimen. At 72 h, the cfu/mL for all regimens were >5 log₁₀ lower than for the corresponding growth control (Figure 3-1B). At the end of the treatment period, the maximum difference in cfu/mL between the three dosage regimens was ~1.25 log₁₀ units. The general similarities in time-courses for bacterial response to each regimen are again reflected in small differences in AUBC₀₋₇₂ (Table 3-2).

3.4.3 Emergence of resistance to colistin

For both ATCC 27853 (Figure 3-2) and the clinical isolate 19056 (data not shown), the PAPs after exposure to the conditions within the *in vitro* model for 72 h, but in the absence of colistin (i.e., growth controls), closely matched those observed at time zero (baseline). At baseline or following 72 h incubation in the model for ATCC 27853, no subpopulations able to grow in the presence of 4 mg/L colistin and above were detected; for clinical isolate 19056, the corresponding value was 0.5 mg/L. The AUCPAPs at baseline and 72 h for clinical isolate 19056 were 0.50 ± 0.04 (*n* = 3) and 0.85 ± 0.22 (*n* = 3), respectively.

The emergence of resistance in ATCC 27853 during treatment with colistin is shown in the PAPs (Figure 3-2); also included in the figure are the AUCPAPs. For the 8 hourly dosage regimen, no growth was detected above 4 mg/L colistin at 48 h, whereas by 72 h, growth was detected in the presence of colistin up to 6 mg/L (Figure 3-2A). For the 12 hourly dosage regimen, no growth was detected above 3 mg/L at 48 h; at 72 h, there was a very substantial change in the PAPs (Figure 3-2B) such that ~0.14% of the population was able to grow at 4 mg/L and growth was detected at 10 mg/L. For the 24 hourly dosage regimen, the PAPs curve after 48 h moved to the right and growth was detected in the presence of 5 mg/L colistin (Figure 3-2C). By 72 h, there was evidence of further emergence of resistance with growth detected in the presence of 10 mg/L colistin. For clinical isolate 19056, no growth was detected in the PAPs at any colistin concentration for any of the dosage regimens (data not shown).

3.5 Discussion

A lack of information on the PK and PD of colistin and CMS has led to confusion regarding the optimal dosing schedule (39). The product information for CMS recommends a maximum daily dose of 5 mg/kg/day (colistin base activity) in two to four divided doses in patients with normal renal function (106-107), although once-daily dosing has also been reported recently (387, 389). Simulated regimens in the *in vitro* PK/PD model were chosen based on the PK of colistin generated from CMS in humans with normal renal function (91), and allowed for an unbound fraction of colistin in human plasma of approximately 0.5 (P. J. Bergen, J. Li and R. L. Nation, unpublished results). Thrice daily dosing (8 h dosage interval, Table 3-1) is the regimen most commonly reported in the literature (38, 99, 160, 162, 166-167, 500). A larger unit dose of colistin was administered to simulate a 12 h dosage interval (162) (Table 3-1). A 24 h dosage interval (Table 3.1) simulated a regimen which has recently been used clinically (387, 389) but has no corresponding recommendation for renally healthy patients in the product information.

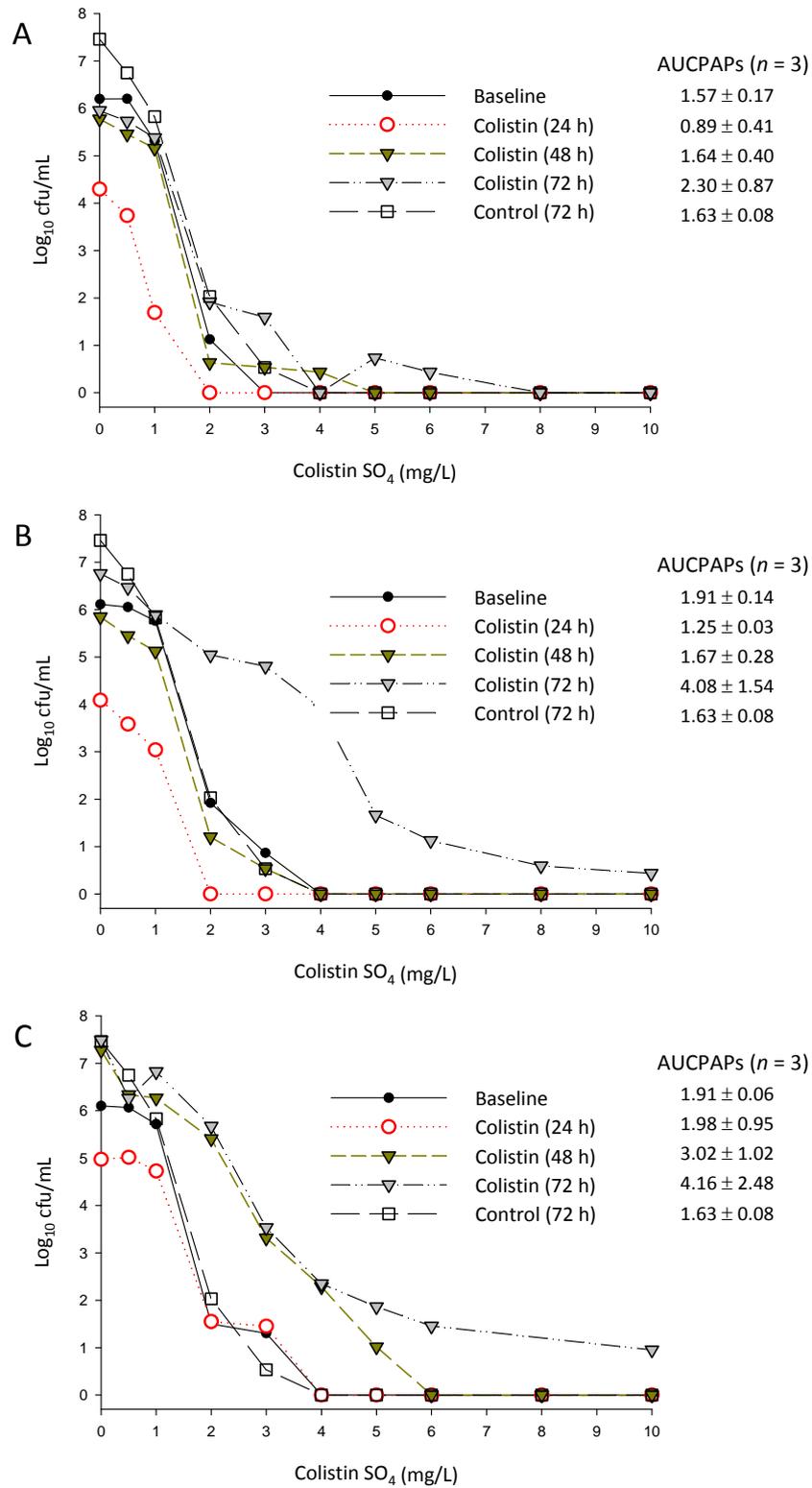


Figure 3-2. PAPs of ATCC 27853 in the *in vitro* PK/PD model: (A) 8 hourly dosing, (B) 12 hourly dosing and (C) 24 hourly dosing.

Both bacterial strains were susceptible to colistin prior to drug exposure (MICs 1 and 0.5 mg/L for ATCC 27853 and 19056, respectively), and each dosage regimen produced colistin concentrations that exceeded the MIC for substantial percentages of the dosage interval (Table 3-1). With each strain, the first exposure to colistin caused rapid and extensive killing to the limit of detection (Figure 3-1). However, regrowth was observed with all regimens. For ATCC 27853, regrowth was detected with each regimen no later than 6 h after the initial administration of colistin, despite the concentrations at this time (~1.0, 1.6 and 3.2 mg/L for 8, 12 and 24 hourly dosage regimens, respectively) remaining at or above the MIC of 1 mg/L. For the clinical isolate 19056, the initial killing activity of colistin was more sustained, which is consistent with its lower MIC (0.5 mg/L). Simulated colistin concentrations with the 8 and 12 hourly dosage regimens remained above the MIC for this isolate throughout the treatment period (Table 3-1), whereas with the 24 hourly regimen the MIC was exceeded for ~17 h (70%) of the dosage interval; the pattern of regrowth seen with this regimen, however, was not dissimilar to that of the other regimens. Indeed, regrowth with the 24 hourly regimen was detected 6 h after the 48 h dose, when colistin concentrations were significantly above the MIC (~6.4× MIC). Thus, regrowth of the clinical isolate occurred with all regimens in the presence of colistin concentrations above the MIC, as was the case with the reference strain.

For each strain, overall bacterial killing and regrowth throughout the experimental period were generally similar among the three regimens (Figure 3-1 and Table 3-2). The AUC/MIC ratios for each strain were similar across each regimen, while the corresponding C_{max}/MIC and $T_{>MIC}$ values differed substantially (Table 3-1). Although this study was not designed to elucidate the PK/PD index most closely related to antibacterial effect of colistin, the similar time-courses of overall bacterial numbers suggests that AUC/MIC is likely to be more important than C_{max}/MIC and $T_{>MIC}$. The same conclusion was reached for polymyxin B from studies conducted in an *in vitro* PK/PD model with once-, twice- and thrice-daily dosing against *P. aeruginosa* (74). Appropriately

designed studies will be required to differentiate more definitively among the three PK/PD indices as the determinant of overall bacterial effect.

The similarity of the time-courses of overall bacterial numbers across the regimens for a given strain may lead to the conclusion that the three regimens were equally effective. The PAPs, however, provided very important information on the relative emergence of resistance across the treatment period with the three regimens. With PAPs, the similarity of the profiles generated in control groups for both strains at baseline and 72 h demonstrated incubation in the *in vitro* model in the absence of colistin did not appreciably alter the proportion of resistant subpopulations. In contrast to growth controls, the proportion of resistant subpopulations present in the reference strain following colistin administration varied with both time and regimen. We recognise that interpretation of PAPs may be influenced by inoculum. As the bacterial numbers at 24 h with each regimen were substantially lower than for any other PAP samples, it is not possible to make meaningful comparison between this and other time points. In contrast, in those cases where there was a substantial change in the PAPs, indicated by a 'shift to the right' and reflected by increases in AUCPAPs, the PAP inoculum was close to that of the corresponding growth controls. At 48 h, resistant subpopulations were found only with the 24-hourly dosage regimen, where growth was detected at 5 mg/L colistin (Figure 3-2C); the ratios of AUCPAP for the 8, 12 and 24 hourly dosage regimens to the AUCPAP for growth control at 48 h (data not shown in Figure 3-2) were 1.06, 1.08 and 1.95, respectively. By 72 h, resistant subpopulations were present with each regimen, but to a lesser extent with the conventional 8 h dosage interval (AUCPAP ratios of 1.41, 2.50 and 2.55 for 8, 12 and 24 h dosage intervals, respectively). Due to the low bacterial numbers present at 24, 48 and 72 h for the clinical isolate, it was not possible to use PAPs to determine whether resistant subpopulations emerged.

Although the three colistin regimens led to generally similar patterns of overall bacterial numbers across the 72 h treatment period, the PAPs for ATCC 27853 revealed that the emergence of resistant subpopulations increased as the dosage interval for colistin increased. Tam *et al.* (74) examined the antibacterial effect of polymyxin B against *P. aeruginosa* using three dosage regimens analogous to those of the present study (once, twice and thrice daily) in an *in vitro* PK/PD model. Although the extent of overall regrowth after 4 days of polymyxin B dosing was similar for each regimen, the proportion of the total population that was resistant (defined as ability to grow at 3× MIC) was substantially lower for the thrice-daily regimen compared with the other two regimens. Although the latter observation was not commented upon (74), it is in agreement with the findings of the present study with colistin, i.e. that a longer dosage interval is associated with greater emergence of resistant subpopulations.

Despite exhibiting concentration-dependent killing, colistin possesses little or no post-antibiotic effect (PAE) at clinically relevant concentrations (63). In the present study, as the dosage interval increased, colistin concentrations remained above the MIC for a smaller proportion of the treatment period (Table 3-1). For ATCC 27853, colistin concentrations with the 8, 12 and 24 hourly dosage regimens remained above the MIC for ~80%, 72% and 53% of the 72 h treatment period, respectively. With this strain, although the time-course of bacterial numbers were generally similar among the three regimens, in the two regimens which employed the greater dosage intervals (12 and 24 h), the emergence of resistance, as revealed by PAPs, was substantially greater and occurred earlier than for the conventional 8 hourly regimen (Figure 3-2). It is also noteworthy that the bacterial load at 24, 48 and 72 h (the only common pre-dose sampling time across the three regimens) was greater with the 24 hourly dosage regimen than the other regimens (Figure 3-1A). In the absence of a substantial PAE, the emergence of resistant subpopulations appears to be favoured by extended dosage intervals leading to protracted periods of colistin concentrations below the MIC. This is an important observation given recent

reports involving administration of CMS in higher, less frequent doses (387, 389) and would suggest that moves towards 24 h and other extended dosage intervals may be detrimental.

In conclusion, the emergence of resistance to colistin is of great concern given CMS is often the last available therapeutic option for treatment of infections caused by MDR Gram-negative bacteria. By simulating the PK of colistin formation in humans administered CMS, we have shown little difference in the overall pattern of bacterial killing and regrowth between three clinically relevant dosage regimens. However, we have also shown that dosing regimens incorporating higher doses of colistin administered less frequently produced greater emergence of resistance than the conventional thrice-daily regimen. This sends a strong warning about the potential negative consequences of moving prematurely to extended-interval dosing. Future studies are warranted to define the prevalence of strains in which resistance is likely to be selected by such dosage regimens. In addition, it will be important to identify the primary PK/PD index determining efficacy and preventing the emergence of resistance.

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3.8 Transparency declaration

We do not have any financial, commercial or proprietary interest in any drug, device or equipment mentioned in this paper.

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature of my contribution to this manuscript included the following:

- design of the study;
- all laboratory experiments;
- data analysis and interpretation;
- preparation of the initial draft of the manuscript and subsequent revisions; and
- formulation of the conclusions and hypothesis arising from the results of the study.

The extent of my contribution was 70%. The following co-authors contributed to the work:

Name	Nature of contribution
Jurgen B. Bulitta	<ul style="list-style-type: none">• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;• review of manuscript drafts and revisions; and• formulation of conclusions and hypotheses arising from the results of the study.
Alan Forrest	<ul style="list-style-type: none">• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;• review of manuscript drafts and revisions; and• formulation of conclusions and hypotheses arising from the results of the study.
Brian T. Tsuji	<ul style="list-style-type: none">• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;• review of manuscript drafts and revisions; and• formulation of conclusions and hypotheses arising from the results of the study.
Jian Li	<ul style="list-style-type: none">• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;• review of manuscript drafts and revisions; and• formulation of conclusions and hypotheses arising from the results of the study.
Roger L. Nation	<ul style="list-style-type: none">• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;• review of manuscript drafts and revisions; and• formulation of conclusions and hypotheses arising from the results of the study.

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Chapter Four

Pharmacokinetic/pharmacodynamic investigation of colistin against

Pseudomonas aeruginosa using an *in vitro* model

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4.1 Abstract

Colistin plays a key role in treatment of serious infections by *Pseudomonas aeruginosa*. The aims of this study were to (i) identify the pharmacokinetic/pharmacodynamic (PK/PD) index (i.e., the area under the unbound concentration-time curve to MIC ratio [$fAUC/MIC$], the unbound maximal concentration to MIC ratio [fC_{max}/MIC], or the cumulative percentage of a 24-h period that unbound concentrations exceed the MIC [$fT_{>MIC}$]) that best predicts colistin efficacy and (ii) determine the values for the predictive PK/PD index required to achieve various magnitudes of killing effect. Studies were conducted in a one-compartment *in vitro* PK/PD model for 24 h using *P. aeruginosa* ATCC 27853, PAO1, and the multidrug-resistant mucoid clinical isolate 19056 muc. Six intermittent dosing intervals, with a range of fC_{max} colistin concentrations, and two continuous infusion regimens were examined. PK/PD indices varied from 0.06 to 18 for targeted fC_{max}/MIC , 0.36 to 312 for $fAUC/MIC$, and 0 to 100% for $fT_{>MIC}$. A Hill-type model was fit to killing effect data, which were expressed as \log_{10} ratio of the area under the cfu/mL curve for treated regimens versus control. With fC_{max} values equal to or above the MIC, rapid killing was observed following the first dose; substantial regrowth occurred by 24 h with most regimens. The overall killing effect was best correlated with $fAUC/MIC$ ($R^2 = 0.931$) compared to fC_{max}/MIC ($R^2 = 0.868$) and $fT_{>MIC}$ ($R^2 = 0.785$). The magnitudes of $fAUC/MIC$ required for 1- and 2- \log_{10} reductions in the area under the cfu/mL curve relative to growth control were 22.6 and 30.4, 27.1 and 35.7, and 5.04 and 6.81 for ATCC 27853, PAO1 and 19056 muc, respectively. The PK/PD targets identified will assist in designing optimal dosing strategies for colistin.

4.2 Introduction

Globally there is a growing threat from the emergence of multidrug-resistant (MDR) microorganisms (13), especially among a number of important Gram-negative bacterial pathogens (13, 57-58). Colistin (polymyxin E) still retains significant activity against many of these MDR Gram-negative pathogens, including *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae*, which often leaves it the only therapeutic option available (39, 47). With very few new chemical entities against Gram-negative infections in the drug development pipeline (13, 58-59), particularly against *P.aeruginosa* (13), the use of colistin, a once neglected antibiotic, has increased dramatically over the last five years (38-39).

Colistin is available commercially as colistin sulphate (hereafter referred to as colistin) and sodium colistin methanesulphonate (CMS), which is administered parenterally. CMS is an inactive prodrug of colistin (515) and, after parenteral administration, colistin is formed *in vivo* (91-92, 95). Despite its new found importance in therapy, there is a dearth of information on the pharmacokinetic (PK) and pharmacodynamic (PD) properties of colistin, a situation of significant concern given that resistance to colistin is beginning to emerge (39, 45, 61-63). Thus, the aims of the present study were to utilise an *in vitro* PK/PD model to: (i) identify the PK/PD index (i.e., the area under the unbound concentration-time curve to MIC ratio [$fAUC/MIC$], the unbound maximal concentration to MIC ratio [fC_{max}/MIC], or the cumulative percentage of a 24-h period that unbound concentrations exceed the MIC [$fT_{>MIC}$]) that best predicts colistin efficacy and (ii) determine the magnitude of the predictive PK/PD index required to achieve various magnitudes of killing effect.

4.3 Materials and Methods

4.3.1 Bacterial strains and media

Three strains of *P. aeruginosa* were used in the present study: two reference strains, ATCC 27853 and PAO1 (American Type Culture Collection, Rockville, MD), and a MDR mucoid clinical isolate, 19056 muc. The MICs of colistin, as determined by broth microdilution (516), were 1 mg/L for ATCC 27853 and PAO1 and 0.5 mg/L for 19056 muc. All MIC determinations were performed in three replicates on separate days. Storage was in tryptone soy broth (Oxoid, Basingstoke, Hampshire, England) with 20% glycerol (Ajax Finechem, Seven Hills, New South Wales, Australia) at -80°C in cryovials (Simport Plastics, Boloeil, Quebec, Canada).

4.3.2 Chemicals and reagents

Colistin sulphate was purchased from Sigma-Aldrich (lot 095K1048, 20,195 units/mg; St Louis, MO). Immediately prior to each experiment, colistin stock solutions were prepared using Milli-Q water (Millipore Australia, North Ryde, New South Wales, Australia), sterilised by filtration with a 0.22-µm-pore-size Millex-GP filter (Millipore, Bedford, MA), and then stored at 4°C before use; colistin is stable under these conditions (90). All other chemicals were from suppliers previously described (80).

4.3.3 Binding of colistin in growth medium

The binding of colistin in cation-adjusted Mueller-Hinton broth (CAMHB, Ca²⁺ at 23.0 mg/L, Mg²⁺ at 12.2 mg/L; Oxoid, Hampshire, England) was measured by equilibrium dialysis using a Perspex dialysis cell unit containing two chambers (1 mL in each chamber) separated by a semipermeable membrane (Spectra/Por-2, lot 29300; Spectrum Laboratories, Rancho Dominguez, CA). Colistin (sulphate) was spiked into CAMHB (donor chamber) to achieve concentrations of 10 and 30 mg/L and dialysed at 37°C against the same volume of isotonic phosphate buffer (0.067 M, pH 7.3) (acceptor chamber); samples were prepared in triplicate. Samples of CAMHB and buffer were

removed from each reservoir after 24 hours (shown in preliminary studies to be the time required for equilibration) and stored at -80°C until analysed as described below. The fraction of colistin unbound in CAMHB (f_u) was calculated as follows: (acceptor colistin concentration)/(donor colistin concentration).

4.3.4 *In vitro* PK/PD model and colistin dosing regimens

Experiments to examine the PK/PD indices driving the microbiological response to colistin were conducted over 24 h using a one-compartment *in vitro* PK/PD model (517). Briefly, the system consisted of four sealed containers (compartments), each containing 100 mL of CAMHB at 37°C and a magnetic stir bar to ensure adequate mixing. One compartment acted as a control to define growth dynamics in the absence of colistin, whereas colistin was delivered into the remaining compartments to achieve the desired intermittent injection or continuous infusion regimens (see below).

Prior to each experiment, strains were subcultured onto horse blood agar (Media Preparation Unit, The University of Melbourne, Parkville, Australia) and incubated at 35°C overnight. One colony was then selected and grown overnight in 10 mL of CAMHB, from which early-log-phase growth was obtained. A 1.0-mL aliquot of this early-log-phase bacterial suspension was inoculated into each compartment at the commencement of each experiment to yield approximately 10^6 cfu/mL.

Both intermittent and continuous infusion dosage regimens of colistin were examined. For dosage regimens involving intermittent administration of colistin, sterile drug-free CAMHB from a central reservoir was pumped through the system at a predetermined rate, displacing CAMHB from each compartment, thus simulating colistin elimination (half-life $[t_{1/2}] = 4$ h) in healthy volunteers (518) and people with cystic fibrosis (91, 99, 412). Flow rates were calibrated prior to experiments and monitored throughout to ensure the system was performing optimally. The

appropriate loading dose of colistin (sulphate) was injected into each treatment compartment following bacterial inoculation to achieve the desired steady-state C_{\max} ($\cong fC_{\max}$; see Results); intermittent maintenance doses were given at appropriate intervals to achieve the same fC_{\max} as after the respective loading dose. This simulated steady-state PK with intermittent dosing. For the continuous-infusion regimens, colistin was spiked into the CAMHB within the central reservoir prior to initiation of the experiment such that all media flowing through the system (with the exception of the growth control compartment) contained a constant concentration of colistin. For both intermittent and continuous regimens, serial samples were collected aseptically, as shown Table 4-1, for viable counting and determination of colistin concentrations. Viable counting was performed using a Whitley automatic spiral plater (WASP; Don Whitley Scientific, West Yorkshire, United Kingdom) and a ProtoCOL colony counter (Synbiosis, Cambridge, United Kingdom); the limits of counting and quantification of the procedure were 20 and 400 cfu/mL, respectively, as specified in the ProtoCOL manual.

Dosing regimens were selected to maximally differentiate among the PK/PD indices under investigation ($fAUC/MIC$, fC_{\max}/MIC and $fT_{>MIC}$). Overall, six intermittent dosing intervals (every 3, 4, 6, 8, 12, and 24 h) were examined with fC_{\max} varied across each schedule; two continuous infusion (CI) regimens were also examined (Table 4-1). In all, 85 treatments across 37 different combinations of dosage frequency and fC_{\max} were examined for the three strains. PK/PD indices varied from 0.06 to 18 for targeted fC_{\max}/MIC , 0.36 to 312 for $fAUC/MIC$, and from 0 to 100% for $fT_{>MIC}$. The range of fC_{\max} used extended to greater than that seen in humans (91) to explore the complete dose-response relationship from essentially no effect to maximum effect.

Table 4-1. Colistin dosage regimens and sampling times in the *in vitro* PK/PD model^a

Parameter	Dosage regimen ^b					
	3 h	4 h	8 h	12 h	24 h	CI
Target fC_{\max} (mg/L)						
ATCC 27853	0.50, 1.5	1.0, 2.0, 3.5, 9.0, 18	3.0*	2.0, 4.5*, 9.0	0.20, 0.25, 0.30, 0.50, 1.0, 1.5, 3.5, 9.0*, 18	1.0, 4.5
PAO1			3.0	3.0, 9.0, 18	0.06, 0.13, 0.25, 0.50, 1.0, 2.0	1.0
19056 muc ^c			3.0*	0.06, 0.13, 0.15, 0.03, 1.0, 1.5, 4.5*	0.03, 0.06, 0.13, 0.15, 0.25, 0.35, 0.50, 1.0, 1.5, 2.0, 9.0*	
Sampling times (h) for microbiological measurements	0, 1, 2, 3, 4, 6, 7, 9, 10, 12, 13, 15, 16, 18, 19, 21, 22, 24	0, 1, 2, 4, 5, 6, 8, 9, 12, 13, 16, 17, 20, 21, 24	0, 1, 2, 4, 6, 8, 9, 16, 17, 24	0, 1, 2, 4, 6, 8, 12, 13, 24	0, 1, 2, 4, 6, 8, 24	0, 1, 2, 4, 6, 8, 12, 24

^a Dosage regimens involved intermittent administration at the dosage intervals indicated (3 to 24 h) to achieve target fC_{\max} or constant concentrations simulating continuous infusion (CI).

^{b,*}, results taken from the first 24 h of a previous study (517).

^c For this strain, an additional 6-hourly regimen with an fC_{\max} of 1 mg/L was performed.

4.3.5 Quantification of colistin in CAMHB and buffer

Samples (250 μL) collected from the *in vitro* PK/PD model and equilibrium dialysis experiments were analysed as previously described (517). Concentrations of colistin were measured using high-performance liquid chromatography (HPLC) with derivatisation and fluorescence detection (417) with an assay range for colistin sulphate of 0.10 to 6.00 mg/L; samples were diluted when the expected colistin concentrations were higher than the upper limit of quantification. Analysis of quality control (QC) samples with nominal concentrations of 0.40, 4.00, 9.00 and 18.00 mg/L (the latter two QC samples required dilution) demonstrated that the accuracy and coefficients of variation were within 15%.

4.3.6 Determination of predictive PK/PD index

The following PK/PD indices were determined for each dosage regimen: $f\text{AUC}/\text{MIC}$, $fC_{\text{max}}/\text{MIC}$ and $fT_{>\text{MIC}}$. The area under the unbound colistin concentration-versus-time curves over 24 h ($f\text{AUC}$; $\mu\text{g}\cdot\text{h}/\text{mL}$) was determined by equation 1 (see below), where n is the number of dosing intervals in the 24-h period, and k is the elimination rate constant (0.17 h^{-1} , corresponding to a 4-h half-life). The percentage of time that unbound concentrations exceeded the MIC ($fT_{>\text{MIC}}$) was determined by equation 2. Targeted fC_{max} , trough (fC_{min}), and k values were used for all calculations.

$$f\text{AUC} = n \cdot (fC_{\text{max}} - fC_{\text{min}})/k \quad (1)$$

$$fT_{>\text{MIC}} = n \cdot \ln(fC_{\text{max}}/\text{MIC})/k/24 \cdot 100\% \quad (2)$$

The area under the curve (AUC_{cfu}) of the time-course profile of bacterial numbers (cfu/mL from 0 to 24 h) was calculated using the linear trapezoidal rule. The killing effect (drug effect) chosen as the measure of efficacy (E) was quantified by the log ratio area method, which compensates for bacterial loss from our model (519):

$$E = \log_{10} \frac{\text{AUC}_{\text{cfu (Treatment)}}}{\text{AUC}_{\text{cfu (Growth control)}}} \quad (3)$$

The relationship between killing effect (E) and each of the three PK/PD indices was analysed by using the Hill equation with a baseline and an inhibitory effect:

$$E = E_0 - \frac{E_{\max} \cdot x^{\gamma}}{\text{EI}_{50}^{\gamma} + x^{\gamma}} \quad (4)$$

where E is the observed effect, E_0 is the baseline effect in the absence of colistin, E_{\max} is the maximal effect, x is the PK/PD index under investigation, EI_{50} is the magnitude of the PK/PD index producing 50% of E_{\max} , and γ is the sigmoidicity coefficient (Hill's constant).

The parameters of equation 4 were estimated by three different approaches: (i) uniformly weighted least-squares estimation in WinNonlin Professional (version 5.2.1; Pharsight Corp., Mountain View, CA), (ii) a pooled fitting approach based on maximum-likelihood estimation in NONMEM VI (level 1.2), and (iii) nonlinear mixed-effects modeling in NONMEM VI using the first-order conditional estimation method. An additive error model on a log scale was used for approaches ii and iii. The data for all regimens were fit separately for each of the 9 combinations of strains and PK/PD indices for approaches i and ii. The data for all regimens and all three strains were comodeled for approach iii for each of the PK/PD indices. Median estimates and 90% nonparametric confidence intervals (5% to 95% percentile) were determined via nonparametric bootstrapping as described previously using 1,000 replicates for each analysis of approaches ii and iii (520). For each bootstrap dataset, 44 regimens were randomly chosen for strain ATCC 27853, 26 regimens were randomly chosen for 19056 muc and 15 regimens were randomly chosen for PAO1. The P values for two-sided nonparametric comparisons between PK/PD indices were computed based on the pairwise differences in objective function values between two PK/PD indices for each of the 1,000 bootstrap replicates. Determination of the

PK/PD index best characterising killing effect was assessed by the coefficient of determination (R^2), NONMEM's objective function ($-2 \cdot \log$ -likelihood), and visual inspection of the observed versus fitted effect plots.

The drug exposure ($x_{nn \log_{10} \text{ effect}}$) required for 1- or 2- \log_{10} reduction in the area under the cfu/mL curve relative to growth control (equation 5) and the drug exposure (EI_{90}) causing 90% of maximal effect (equation 6) were calculated as follows:

$$x_{nn \log_{10} \text{ reduction}} = \frac{EI_{50}}{\left(\frac{E_{\max}}{nn} - 1\right)^{\frac{1}{\gamma}}} \quad (5)$$

$$EI_{90} = \frac{EI_{50}}{\left(\frac{1}{0.9} - 1\right)^{\frac{1}{\gamma}}} \quad (6)$$

The desired extent of \log_{10} reduction (nn) enters equation 5 as a positive number. Equation 5 only yields exposure targets only if E_{\max} is larger than nn .

4.4 Results

4.4.1 Binding of colistin in CAMHB

The fraction of colistin unbound in CAMHB (f_u) at equilibrium, with initial concentrations for colistin sulphate of 10 and 30 mg/L, was 0.96 and 0.95, respectively, indicating practical equivalence of total and unbound concentrations.

4.4.2 PK validation

The mean \pm standard deviation (SD; $n = 58$) of the absolute percentage relative differences between targeted and achieved colistin fC_{\max} concentrations as determined by HPLC was 9.76 ± 14.2 , and the mean of the percentage relative differences was -4.64 ± 16.7 . The observed mean $t_{1/2}$ for the simulated intermittent dosage regimens was 4.06 ± 0.46 h ($n = 47$) for the targeted value of 4 h; since the fC_{\min} for some dosage regimens was below the lower limit of quantification of the HPLC assay (0.10 mg/L), $t_{1/2}$ was not directly measured in all experiments.

4.4.3 Bacterial killing of *P. aeruginosa* in the *in vitro* PK/PD model

Representative killing profiles for each strain are shown in Figure 4-1. The initial inocula in control and treatment compartments (mean \pm the SD) were: 6.21 ± 0.09 ($n = 15$) and 6.18 ± 0.14 ($n = 44$) \log_{10} cfu/mL for ATCC 27853, 6.39 ($n = 2$) and 6.29 ± 0.13 ($n = 15$) \log_{10} cfu/mL for PAO1, and 5.88 ± 0.41 ($n = 4$) and 6.08 ± 0.32 ($n = 26$) \log_{10} cfu/mL for 19056 muc. After 24 h, bacterial numbers in control compartments had increased to 8.06 ± 0.20 ($n = 15$) \log_{10} cfu/mL for ATCC 27853, 8.21 ($n = 2$) \log_{10} cfu/mL for PAO1, and 7.74 ± 0.07 ($n = 4$) \log_{10} cfu/mL for 19056 muc. For all strains there was early dosage-dependent killing followed by regrowth to various extents (Figure 4-1).

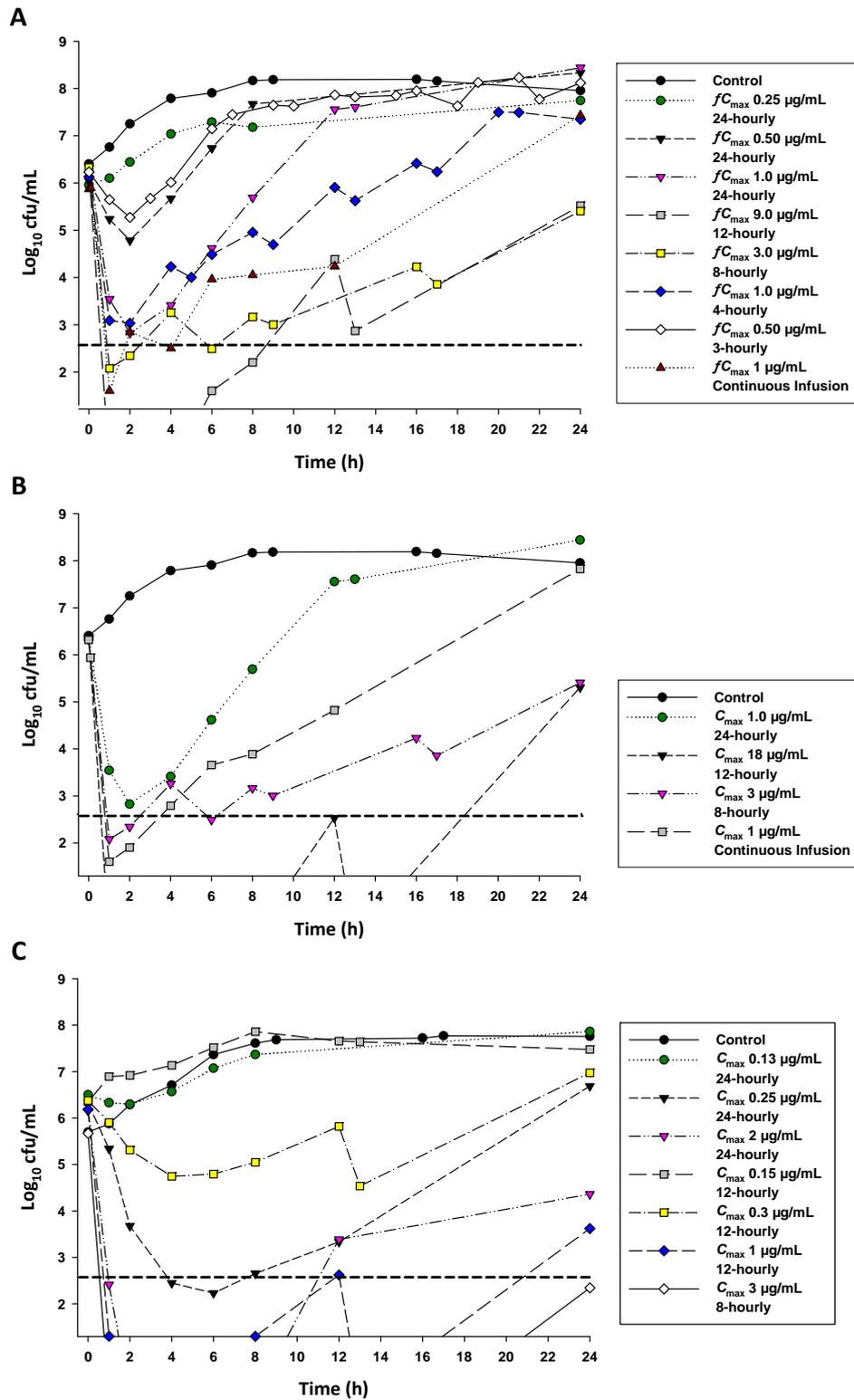


Figure 4-1. Typical microbiological responses observed in the *in vitro* PK/PD model simulating the colistin pharmacokinetics of different dosage regimens using ATCC 27853 (MIC = 1 mg/L) (A), PAO1 (MIC = 1 mg/L) (B), and the MDR clinical isolate 19056 muc (MIC = 0.5 mg/L) (C). The y axis starts from the limit of counting, and the limit of quantification (LOQ) is indicated by the horizontal broken line.

4.4.4 Relationships between killing effect and PK/PD indices

Since the differences in initial inocula were small (see above) we elected not to standardise AUC_{cfu} by dividing by initial inoculum when calculating the killing effect. Parameter estimates for modeling approaches i, ii, and iii were consistent for all three PK/PD indices and robust for $fAUC/MIC$ and fC_{max}/MIC ; approach iii yielded the most robust estimates for $fT_{>MIC}$. Modeling by approach iii (Table 4-2) indicated that between-strain variability was largest for EI_{50} and negligible for the other parameters. The confidence intervals for EI_{50} indicated significantly lower values for strain 19056 muc. The relationships between killing effect and $fAUC/MIC$, fC_{max}/MIC or $fT_{>MIC}$ are shown in Figure 4-2. Of the three indices, $fAUC/MIC$ best described the killing effect ($R^2 = 0.931$; Figure 4-2A); the relationship between the killing effect and fC_{max}/MIC had a lower R^2 of 0.868 (Figure 4-2B). A poorer relationship existed between the killing effect and $fT_{>MIC}$, where a high degree of scatter and systematic deviations from the curve fit was observed ($R^2 = 0.785$; Figure 4-2C). Median (nonparametric 90% confidence interval) objective function values from modeling approach iii were -79.6 (-111 to -54.4) for $fAUC/MIC$, -28.3 (-72.1 to 2.9) for fC_{max}/MIC , and 16.4 (-35.9 to 47.5) for $fT_{>MIC}$. The objective function was significantly lower for $fAUC/MIC$ compared to fC_{max}/MIC ($P = 0.050$, two-sided testing; from 1,000 nonparametric bootstrap replicates) and for $fAUC/MIC$ compared to $fT_{>MIC}$ ($P < 0.01$). Differences between fC_{max}/MIC and $fT_{>MIC}$ were not significant ($P = 0.2$).

The magnitudes of the $fAUC/MIC$ index required for 1- and 2- \log_{10} reduction in the area under the cfu/mL curve relative to growth control for each strain are shown in Table 4-3. Near-maximal killing was achieved with $fAUC/MIC$ ratios of approximately 40, 50 and 9 for ATCC 27853, PAO1, and 19056 muc, respectively (Table 4-3 and Figure 4-2A).

Table 4-2. Median parameter estimates from 1,000 bootstrap replicates for each of the three PK/PD indices^a

PK/PD index	Strain	Median parameter estimates (90% nonparametric confidence intervals)			
		E_0	E_{max}	El ₅₀	γ
$fAUC/MIC$	ATCC 27853	-0.232 (-0.349 to -0.139)	3.05 (2.88–3.21)	26.4 (23.8–28.9)	4.77 (3.20–7.27)
	PAO1			31.2 (28.6–35.4)	
	19056 muc			5.91 (4.60–12.5)	
fC_{max}/MIC	ATCC 27853	-0.110 (-0.319 to 0.025)	3.12 (2.81–3.38)	1.82 (1.54–2.25)	3.13 (2.08–12.2)
	PAO1			2.48 (1.74–3.27)	
	19056 muc			0.834 (0.645–1.27)	
$fT_{>MIC}$	ATCC 27853	-0.365 (-0.558 to -0.212)	3.07 (2.68–3.39)	39.6 (31.9–47.1)	2.13 (1.26–4.30)
	PAO1			68.4 (41.0–135)	
	19056 muc			13.6 (9.90–21.7)	

^a Data for all three strains were comodeled for each PK/PD index (approach iii, see Materials and Methods). Initial models with between-strain variability for all four parameters showed that the variability in E_0 , E_{max} and γ was negligible. Since exclusion of the variability for these three parameters did not affect the objective function significantly, the final model only included between strain variability for El₅₀.

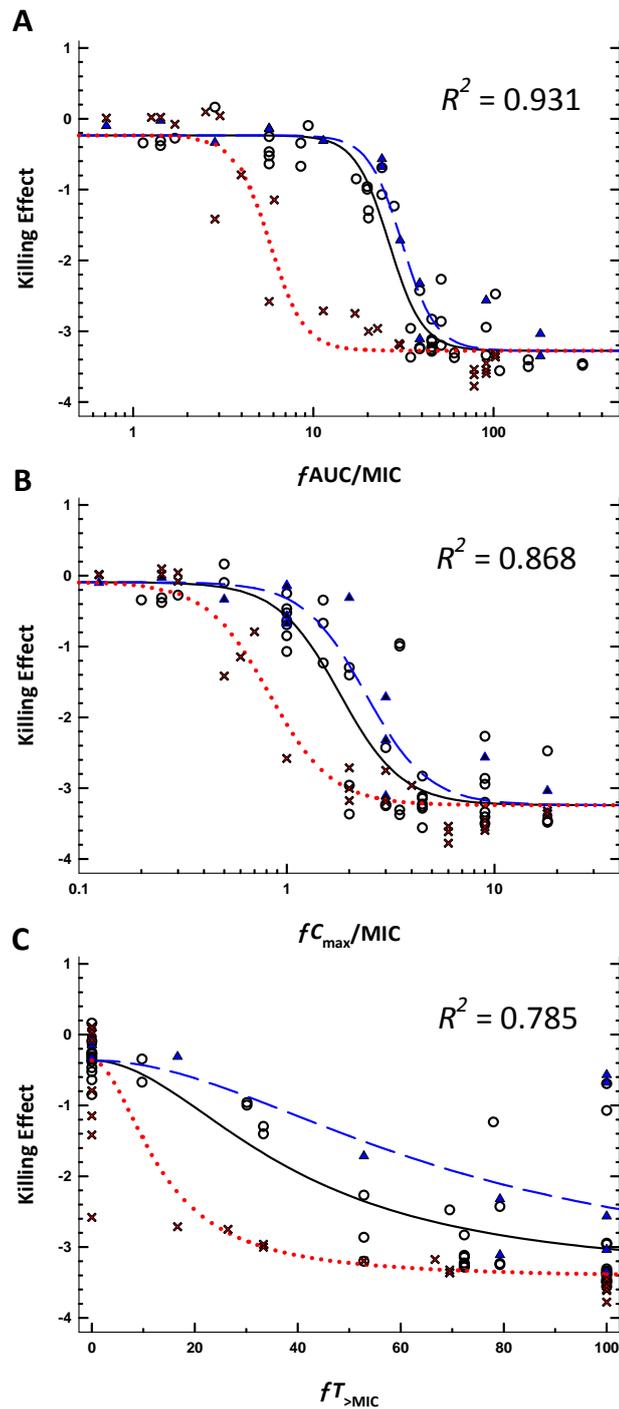


Figure 4-2. Relationship between killing effect (log area ratio) against *P. aeruginosa* ATCC 27853 (black solid line and open circles), PAO1 (blue dashed line and solid triangles), and 19056 muc (red dotted line and crosses) as a function of three PK/PD indices: $fAUC/MIC$ (A), fC_{max}/MIC (B), and $fT_{>MIC}$ (C). Each data point represents the result from a single treatment run. Lines represent model-generated fits using modeling approach iii (see Materials and Methods and Table 4-2).

Table 4-3. Median target values from 1,000 bootstrap replicates of colistin $fAUC/MIC$ for 1- and 2- \log_{10} reductions in the area under the cfu/mL curve relative to growth control and for 90% (EI_{90}) of maximal effect

Killing effect	Median target values (90% nonparametric confidence intervals)		
	ATCC 27853	PAO1	19056 muc
1- \log_{10} reduction	22.6 (19.9–25.7)	27.1 (23.6–29.9)	5.04 (3.93–10.5)
2- \log_{10} reduction	30.4 (27.2–33.0)	35.7 (32.6–41.7)	6.81 (5.21–14.3)
EI_{90}	42.0 (35.3–52.1)	49.3 (40.8–68.5)	9.78 (6.71–20.3)

4.5 Discussion

Colistin, which first became clinically available more than 50 years ago, was never subjected to many of the drug development procedures required of new drugs today. As a consequence, current dosage regimens for CMS/colistin are chosen empirically, and much is still to be learned about the PK, PD, and the PK/PD index that best correlates with antibacterial activity of colistin (39). Such information is important for rational design of optimal dosing strategies.

Previous animal or *in vitro* pharmacodynamic studies have reported regrowth of *P. aeruginosa* with a range of colistin (395, 517) or polymyxin B (74) dosage regimens. Similarly, this was generally observed in the present study despite unbound colistin concentrations far in excess of clinically achievable concentrations in some experiments. Even for the regimens that achieved very extensive bacterial killing after the first dose, substantially less net killing occurred after subsequent doses (Figure 4-1). We have shown previously the presence of resistant subpopulations after 72-h exposure to colistin in the same *in vitro* PK/PD model (517). We are currently developing a mechanism-based mathematical model that can describe and predict the time course of bacterial growth and killing and which incorporates the emergence of multiple bacterial populations with varying colistin susceptibilities.

Three previous studies have addressed issues around the exposure-response relationships for polymyxins. Using a limited dose fractionation design, Tam *et al.* (74) investigated the PD of polymyxin B against *P. aeruginosa* in an *in vitro* PK/PD hollow-fiber model and suggested that activity was most likely linked to AUC/MIC. The study by Tam *et al.* (74), however, was not specifically designed to examine the relationship between efficacy and each PK/PD index. In a study of colistin against *P. aeruginosa* in a neutropenic mouse infection model, Ketthireddy *et al.* (395) concluded that once-daily dosing was most effective and that the data were consistent with C_{\max}/MIC being the PK/PD index most predictive of efficacy; PK data, however, were not included in that study. In neutropenic mouse thigh and lung infection models, Dudhani *et al.* (402) found that $f\text{AUC}/\text{MIC}$ was the index most predictive of efficacy. In the present study, we used a much larger dose fractionation design in an *in vitro* dynamic model to distinguish between PK/PD indices determining colistin efficacy. A Hill-type model was fit to the data using an area-based method whereby all cfu/mL versus time data for each regimen were taken into account. This approach allowed for a measure of the time-averaged drug effect and has been implemented in a previous investigation with vancomycin against *Staphylococcus aureus* (519). The analysis demonstrated that $f\text{AUC}/\text{MIC}$ was most closely correlated with bacterial killing (Figure 4-2). Estimates of PD parameters were precise and consistent between all three estimation approaches.

In order to design dosage regimens rationally, it is necessary to know not only which PK/PD index is most predictive of bacterial killing but also the magnitude of that index needed to achieve various extents of kill (521). In the present study, respective values of $f\text{AUC}/\text{MIC}$ of ~25 and 35 for the reference strains were required to achieve 1- and 2-log reductions in the area under the cfu/mL curve relative to growth control (Table 4-3). These results are in extremely good agreement with those obtained by Dudhani *et al.* (402) in the neutropenic mouse thigh infection model against the same two strains ($f\text{AUC}/\text{MIC}$ values of ~23 and 34 for 1- and 2-log reductions, respectively). Interestingly, the corresponding $f\text{AUC}/\text{MIC}$ values for the MDR clinical isolate were somewhat

lower in our *in vitro* model compared to the neutropenic mouse thigh infection model (~6 and 7 *in vitro* compared with ~16 and 28 *in vivo* for 1- and 2-log reductions, respectively). The explanation for this difference is not known but may relate to differences in growth dynamics of this mucoid strain between *in vitro* and *in vivo* systems. We acknowledge the presence of a washout effect on bacteria with the use of open one-compartment PK/PD systems such as in our study; however, the generally good level of agreement across all strains between the present *in vitro* study and infection models involving neutropenic mice (402) is very reassuring in relation to future clinical applications of the $fAUC/MIC$ targets for colistin.

Unfortunately, it is currently not possible to compare the $fAUC/MIC$ targets from the present and other (402) preclinical studies with the $fAUC/MIC$ values achieved in infected patients receiving currently recommended CMS dosage regimens. The inability to undertake this comparison arises because recent studies have shown that colistin binding in plasma involves the acute-phase reactant α_1 -acid glycoprotein (AAG), and the unbound fraction of colistin is influenced by the concentrations of both colistin and AAG (419). Since plasma AAG concentrations are influenced by pathophysiological stresses including infection (420-421), the f_u of colistin in patients is likely to vary depending on the severity and stage of infection and magnitude of plasma colistin concentration. Although the knowledge of total plasma colistin concentrations achieved in patients is increasing (91-92, 95, 522), there is no information on unbound plasma concentrations. As such information is forthcoming it will be possible to not only assess the ability of current CMS dosage regimens to meet the above-mentioned $fAUC/MIC$ targets but also to design optimised dosage regimens.

The use of once-daily doses of CMS has recently been reported (387, 389), presumably based upon the concentration-dependent killing of colistin observed *in vitro* (63). However, we suggest caution with this approach. First, *in vitro* data suggest that the toxicity of colistin is concentration and time

dependent (436). Moreover, greater nephrotoxicity was observed in rats with a dosage regimen mimicking once-daily dosing of CMS in humans compared to a twice-daily regimen that delivered the same daily dose (390). Second, for both colistin and polymyxin B larger, infrequent doses in *in vitro* models led to greater emergence of resistance in *P. aeruginosa* compared to lower-dose/higher-frequency regimens (74, 517). Third, colistin lacks a significant postantibiotic effect *in vitro* (63, 125), although in a brief report such a phenomenon has been suggested to occur *in vivo* (395); additional studies are needed. Given the recent emergence of resistance to colistin (39, 45, 61-63), the ability to choose regimens which not only maximise killing but also suppress or minimise the development of resistance may prove crucial in preventing this trend.

In the present study, we simulated a 4-h colistin half-life as observed in healthy volunteers (518) and people with cystic fibrosis (91, 99, 412); a longer half-life has been reported in critically-ill patients (92, 95, 523). The PK/PD indices described here are specific for *P. aeruginosa* and may differ for other Gram-negative pathogens; species specific differences in the magnitude of a particular index required to achieve certain levels of killing have been demonstrated for other anti-infectives (376). In addition, *in vitro* PK/PD models lack the defence mechanisms present in patients with intact immune systems; however, they may more adequately reflect drug-related antimicrobial activity in an immunocompromised host. Finally, higher PK/PD target values (e.g., for $fAUC/MIC$) may be required for infections with a high initial inoculum (385).

To our knowledge, this is the first *in vitro* investigation specifically designed to elucidate the relationship between bacterial killing and PK/PD indices for colistin against any organism. We have demonstrated that for colistin $fAUC/MIC$ is the PK/PD index most closely correlated with the killing of *P. aeruginosa*. Our findings are in good agreement with those from recent studies in neutropenic mouse infection models. As information on the pharmacokinetics of unbound colistin in patients is

obtained, the PK/PD targets reported here will assist in designing optimal dosing strategies for this increasingly important therapeutic option.

4.6 Acknowledgements

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The content here 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.

We have no conflicts of interest to declare.

Declaration for Thesis Chapter 5

Declaration by candidate

In the case of Chapter 5, the nature of my contribution to this manuscript included the following:

- design of the study;
- all laboratory experiments;
- data analysis and interpretation;
- preparation of the initial draft of the manuscript and subsequent revisions; and
- formulation of the conclusions and hypothesis arising from the results of the study.

The extent of my contribution was 70%. The following co-authors contributed to the work:

Name	Nature of contribution
Alan Forrest	<ul style="list-style-type: none">• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;• review of manuscript drafts and revisions; and• formulation of conclusions and hypotheses arising from the results of the study.
Jurgen B. Bulitta	<ul style="list-style-type: none">• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;• review of manuscript drafts and revisions; and• formulation of conclusions and hypotheses arising from the results of the study.
Brian T. Tsuji	<ul style="list-style-type: none">• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;• review of manuscript drafts and revisions; and• formulation of conclusions and hypotheses arising from the results of the study.
Hanna Sidjabat	<ul style="list-style-type: none">• performed PCR assays; and• review of manuscript drafts and revisions
David L. Paterson	<ul style="list-style-type: none">• interpretation of PCR results; and• review of manuscript drafts and revisions
Jian Li	<ul style="list-style-type: none">• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;• review of manuscript drafts and revisions; and• formulation of conclusions and hypotheses arising from the results of the study.

Roger L. Nation

- supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;
 - review of manuscript drafts and revisions; and
 - formulation of conclusions and hypotheses arising from the results of the study.
-

Candidate's signature

Date

Phillip J. Bergen		
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Declaration by co-authors

The undersigned hereby certify that:

1. the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors;
2. they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
3. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
4. there are no other authors of the publication according to these criteria;
5. potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit;
and
6. the original data are stored at the Facility for Anti-infective Drug Development and Innovation (FADDI; Monash Institute of Pharmaceutical Sciences), and will be held for at least five years from the date indicated below.

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Chapter Five

Clinically relevant plasma concentrations of colistin in combination with imipenem enhance pharmacodynamic activity against multidrug-resistant *Pseudomonas aeruginosa* at multiple inocula

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5.1 Abstract

Use of combination antibiotic therapy may be beneficial against rapidly emerging resistance in *Pseudomonas aeruginosa*. The aim of this study was to systematically investigate *in vitro* bacterial killing and resistance emergence with colistin alone and in combination with imipenem against multidrug-resistant (MDR) *P. aeruginosa*. Time-kill studies were conducted over 48 h using 5 clinical isolates and ATCC 27853 at two inocula ($\sim 10^6$ and $\sim 10^8$ cfu/mL); MDR, non-MDR, and colistin-heteroresistant and -resistant strains were included. Nine colistin/imipenem combinations were investigated. Microbiological response was examined by log changes at 6, 24, and 48 h. Colistin combined with imipenem at clinically relevant concentrations increased bacterial killing against MDR and colistin-heteroresistant isolates at both inocula. Substantial improvements in activity with combinations were observed across 48 h with all colistin concentrations at the low inoculum and with 4 and 16 \times MIC (or 4 and 32 mg/L) colistin at the high inoculum. Combinations were additive or synergistic against imipenem-resistant isolates (MICs, 16 and 32 mg/L) at the 10^6 -cfu inoculum in 9, 11, and 12 of 18 cases (i.e., 9 combinations across 2 isolates) at 6, 24, and 48 h, respectively, and against the same isolates at the 10^8 -cfu inoculum in 11, 7, and 8 cases, respectively. Against a colistin-resistant strain (MIC, 128 mg/L), combinations were additive or synergistic in 9 and 8 of 9 cases at 24 h at the 10^6 - and 10^8 -cfu inocula, respectively, and in 5 and 7 cases at 48 h. This systematic study provides important information for optimisation of colistin-imipenem combinations targeting both colistin-susceptible and -resistant subpopulations.

5.2 Introduction

The world is facing a growing threat from multidrug-resistant (MDR) Gram-negative ‘superbugs’ such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* (13, 57-58). This problem is compounded by a lack of novel antimicrobial agents in the drug development pipeline for Gram-negative infections (13, 58-59), in particular those caused by *P. aeruginosa* (13), and novel agents with activity against this pathogen may not be available for approximately 10 years (11). This has led to the reevaluation of colistin (also known as polymyxin E), a multicomponent cationic polypeptide antibiotic that entered clinical use in 1959 but was largely replaced by aminoglycosides in the 1970s due to concerns about the potential for nephro- and neuro-toxicity (38-39, 47, 60). Owing to its significant *in vitro* antibacterial activity against Gram-negative ‘superbugs’, colistin is often the only therapeutic option available to treat infections by these pathogens (39, 45, 524), and therefore, its use has increased substantially over the last five years, especially for critically-ill patients (10, 39).

It is now evident that the plasma colistin concentrations achieved in critically-ill patients with the currently recommended dosage regimens are suboptimal for a significant proportion of patients (95, 97). Unfortunately, increasing the daily dose may not be an acceptable option since nephrotoxicity is a dose-limiting adverse effect and occurs in 30 to 50% of patients (97, 224, 235). It is therefore not surprising that suboptimal concentrations cause emergence of resistance to colistin which seriously threatens colistin therapy (525-526). *In vivo* (395, 527) and *in vitro* (517, 528) studies show the potential for the rapid emergence of colistin resistance with monotherapy. The phenomenon of colistin heteroresistance (the presence of colistin-resistant subpopulations in an isolate considered susceptible by MIC measurement) (116) has been reported for *A. baumannii* (116, 529) and *K. pneumoniae* (64, 127, 530) but not yet for *P. aeruginosa*. Heteroresistance very likely contributes to emergence of colistin resistance. 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 imipenem against *P. aeruginosa*. Key aspects of this study were the use of MDR isolates with varying susceptibilities to colistin and imipenem (including colistin-heteroresistant isolates first identified in this study, as well as colistin- and imipenem-resistant strains), examination of combinations of clinically relevant drug concentrations at both low and high inocula, and monitoring of emergence of resistance to colistin with real-time population analysis profiles (PAPs).

5.3 Materials and Methods

5.3.1 Bacterial isolates

Five clinical isolates and *P. aeruginosa* ATCC 27853 (American Type Culture Collection, Manassas, VA) were selected to represent a mixture of strains susceptible and resistant to colistin and imipenem, colistin-heteroresistant and nonheteroresistant strains, and multidrug-resistant (MDR) and non-MDR strains. MDR was defined as diminished susceptibility to at least two of the following five drug classes: antipseudomonal cephalosporins, antipseudomonal carbapenems, β -lactam- β -lactamase inhibitor combinations, antipseudomonal fluoroquinolones, and aminoglycosides (29). In addition, all strains were examined by PCR for the presence of genes encoding cephalosporinases and carbapenemases, i.e. IMP-, VIM-, NDM-, KPC-, CTX-M-, SHV-, and CMY-type β -lactamases (531-532). The isolates are described in detail in Table 5-1. All clinical isolates were collected from patients with cystic fibrosis, had different pulsed-field gel electrophoresis patterns, and were considered unrelated according to the criteria established by Tenover *et al.* (533). The MICs to colistin and imipenem were determined for each isolate in four replicates in cation-adjusted Mueller-Hinton broth (CAMHB; containing 23.0 mg Ca^{2+} /L and 12.2 mg Mg^{2+} /L; Oxoid, Hampshire, England) via broth microdilution (152). Isolates were stored in

tryptone soy broth (Oxoid) with 20% glycerol (Ajax Finechem, Seven Hills, NSW, Australia) at -80°C in cryovials (Simport Plastics, Boloeil, Quebec, Canada).

Table 5-1. MICs for the *P. aeruginosa* isolates used in this study

Isolate	MIC (mg/L) ^a		cephalosporinase and carbapenemase typing	MDR ^b
	Colistin	Imipenem		
ATCC 27853 ^c	1	2	negative	No
19147 n/m	128	4	IMP & CTX-M positive ^d	Yes
19056 muc	0.5	4	negative	Yes
20509 n/m ^c	0.5	1	negative	No
19271 n/m ^c	2	32	negative	Yes
20891 n/m ^c	1	16	negative	Yes

^a CLSI breakpoints for colistin were ≤2 mg/L for susceptibility, 4 mg/L for intermediacy, and ≥8 mg/L for resistance. For imipenem, the breakpoints were ≤4 mg/L for susceptibility, 8 mg/L for intermediacy, and ≥16 mg/L for resistance (152).

^b Defined as diminished susceptibility to ≥2 of the following 5 drug classes: antipseudomonal cephalosporins, antipseudomonal carbapenems, β-lactam-β-lactamase inhibitor combinations, antipseudomonal fluoroquinolones, and aminoglycosides (29).

^c Colistin heteroresistant. Heteroresistance to colistin was defined as the existence, in an isolate for which the colistin MIC was ≤2 mg/L, of subpopulations able to grow in the presence of >2 mg/L colistin (125).

^d Contains genes encoding IMP-type carbapenemase and CTX-M-type ESBL.

5.3.2 Antibiotics

Colistin sulfate (lot 109K1574; 23,251 U/mg) was purchased from Sigma-Aldrich, St Louis, MO. Colistin (sulfate) was employed in the current study because it is the active antibacterial agent formed *in vivo* after administration of its inactive prodrug, colistin methanesulphonate (CMS) (515). Imipenem (Primaxin[®]; batch K5942) was purchased from Merck Sharp and Dohme, NSW, Australia. Stock solutions of each antibiotic were prepared according to the respective manufacturer's instructions immediately prior to each experiment in order to minimise loss from

degradation; then the solutions were sterilised by filtration with a 0.22- μ m-pore-size Millex-GP filter (Millipore, Bedford, MA).

5.3.3 Population analysis profiles

The possible existence of colistin-resistant subpopulations at baseline was determined via population analysis profiles (PAPs) (inoculum, $\sim 10^8$ cfu/mL). Colistin heteroresistance was defined as the existence, in a colistin-susceptible isolate (i.e., MIC, ≤ 2 mg/L) of subpopulations that were able to grow in the presence of >2 mg/L colistin in the PAPs. Samples of bacterial cell suspensions (50 μ L), appropriately diluted with saline, were spirally plated onto Mueller-Hinton agar (Media Preparation Unit, The University of Melbourne, Parkville, Australia) impregnated with colistin (0, 0.5, 1, 2, 3, 4, 6, 8, or 10 mg/L) by using an automatic spiral plater (WASP; Don Whitley Scientific, West Yorkshire, UK). Colonies were counted using a ProtoCOL colony counter (Synbiosis, Cambridge, UK) after 24 h of incubation (48 h for plates with small colonies) at 35°C; the limit of detection was 20 cfu/mL (equivalent to 1 colony per plate), and the limit of quantification (LOQ) was 400 cfu/mL (equivalent to 20 colonies per plate), as specified in the ProtoCOL manual. Real-time PAPs for colistin were also conducted at the end of time-kill studies (see below).

5.3.4 Time-kill studies

To explore the antimicrobial activities of colistin and imipenem combinations, time-kill studies with each antibiotic alone or in combination were conducted on all isolates at two different starting inocula ($\sim 10^6$ and $\sim 10^8$ cfu/mL). For monotherapy with colistin or imipenem, 2-fold multiples of the MIC (0.25 \times to 64 \times MIC) were employed for susceptible isolates. For the colistin-resistant isolate (19147 n/m; MIC, 128 mg/L), a single colistin concentration of 32 mg/L was employed. Imipenem concentrations of 1, 8, and 32 mg/L were used for imipenem-resistant isolates. In combination experiments, both antibiotics were studied at concentrations of 0.5 \times ,

4×, and 16× MIC for susceptible isolates; for resistant isolates, concentrations of 1, 4, and 32 mg/L for colistin and 1, 8, and 32 mg/L for imipenem were employed. In total, nine colistin-imipenem combinations were examined for each isolate at each inoculum.

Prior to each experiment, isolates were subcultured onto horse blood agar (Media Preparation Unit) and were incubated at 35°C overnight. One colony was then selected and grown overnight in 10 mL CAMHB at 37°C; from this colony, an early log-phase culture was obtained. Each antibiotic was added alone or in combination to 20 mL of a log-phase broth culture of approximately 10⁶ or 10⁸ cfu/mL to yield the desired concentrations. Each 20-mL culture was placed in a sterile 50-mL polypropylene tube (Greiner Bio-one) and was incubated in a shaking water bath at 37°C. Serial samples (100 µL) were collected aseptically for viable-cell counting at 0, 0.5, 1, 2, 4, 6, 24, and 48 h and for PAPs at 48 h (see above) for all experiments involving colistin (including combination arms) and for viable-cell counting only at 0, 1, 2, 4, 6, 24, and 48 h for experiments with imipenem alone. Immediately after sampling and serial dilution, 50 µL of the bacterial cell suspension was spirally plated onto nutrient agar with enumeration after 24 h of incubation (48 h for plates with small colonies) as described under 'PAPs' above.

5.3.5 Pharmacodynamic (PD) analysis

Microbiological responses to monotherapy and combination therapy were examined using the log change method, comparing the change in bacterial counts (log₁₀ cfu/mL) from that at 0 h [log₁₀(CFU₀)] to that at a given time (*t*) (6, 24, or 48 h) [log₁₀(CFU_{*t*})] as follows:

$$\text{log change} = \log_{10}(\text{CFU}_t) - \log_{10}(\text{CFU}_0)$$

Single antibiotic or combination regimens causing a reduction of ≥1 log₁₀ cfu/mL from the initial inoculum at 6, 24, or 48 h were considered active. We considered synergy to be indicated by a ≥2-log₁₀ cfu/mL-lower bacterial count with the combination than with its most active component

at the specified time (464); additivity was defined by a 1- to $<2\text{-log}_{10}$ cfu/mL-lower bacterial count with the combination.

5.4 Results

5.4.1 Microbiological response

The various susceptibilities of the isolates to colistin are evident in the PAPs obtained prior to colistin treatment (Figure 5-1). Representative time-kill profiles for colistin and imipenem monotherapy and combination therapy are shown in Figures 5-2 (inoculum, $\sim 10^6$ cfu/mL) and 5-3 (inoculum, $\sim 10^8$ cfu/mL). Log changes in viable cell counts at each inoculum with clinically relevant colistin concentrations are presented in Tables 5-2 and 5-3. Additional time-kill and log change data are presented in Appendix 2. At the 10^6 -cfu/mL inoculum, regrowth was observed to various extents for all susceptible isolates at 48 h with colistin monotherapy with the majority of concentrations. Regrowth with imipenem monotherapy at concentrations of $\geq 4\times$ or $8\times$ MIC was more variable and substantially less for susceptible isolates at 48 h, even when extended-spectrum β -lactamases (ESBLs) were present. An inoculum effect with colistin monotherapy was generally observed (Figures 5-2 and 5-3, left-hand panels). Killing by imipenem was generally slightly slower at the high inoculum than at the low inoculum, although the extents of reduction in bacterial counts (\log_{10} cfu/mL) were comparable at the two inocula (Figures 5-2 and 5-3).

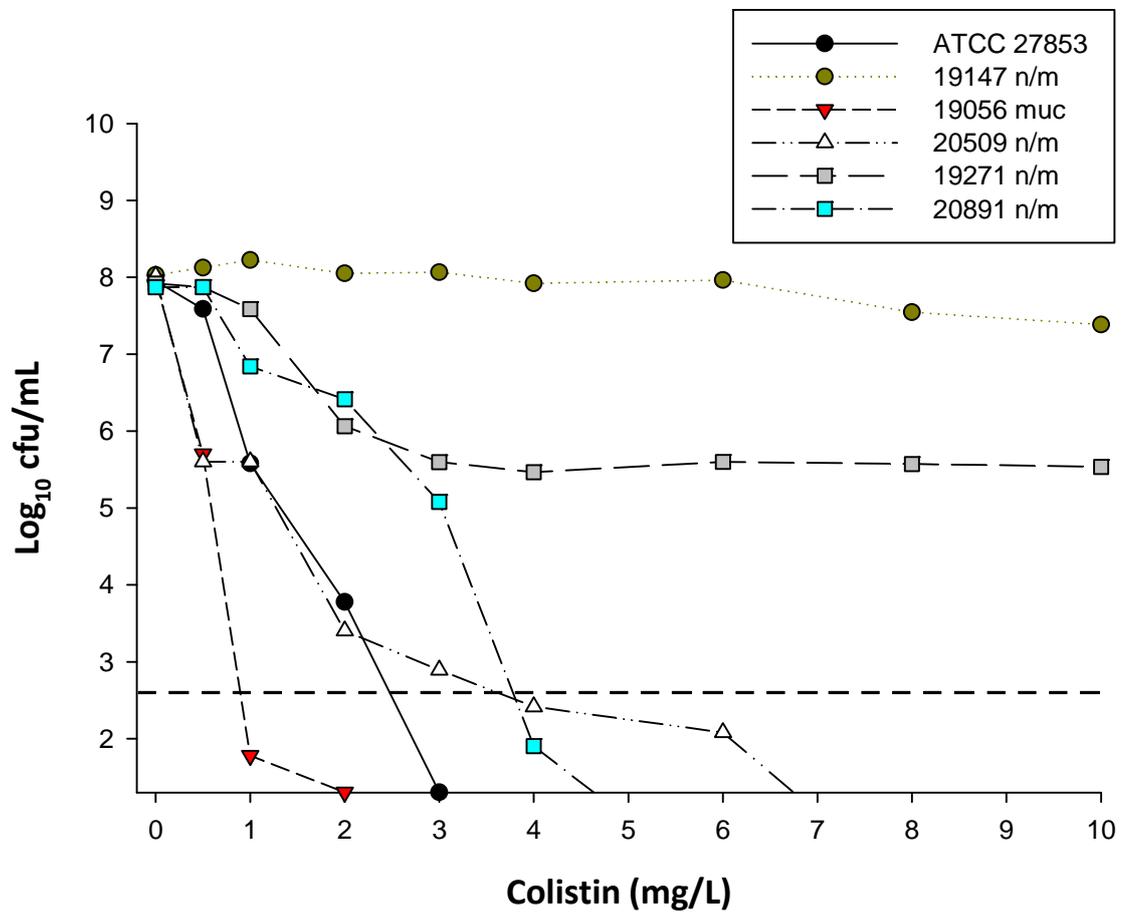


Figure 5-1. Baseline PAPs of the reference strain and all clinical isolates at an initial inoculum of $\sim 10^8$ cfu/mL. The Y-axis starts from the limit of detection and the LOQ is indicated by the dashed horizontal line.

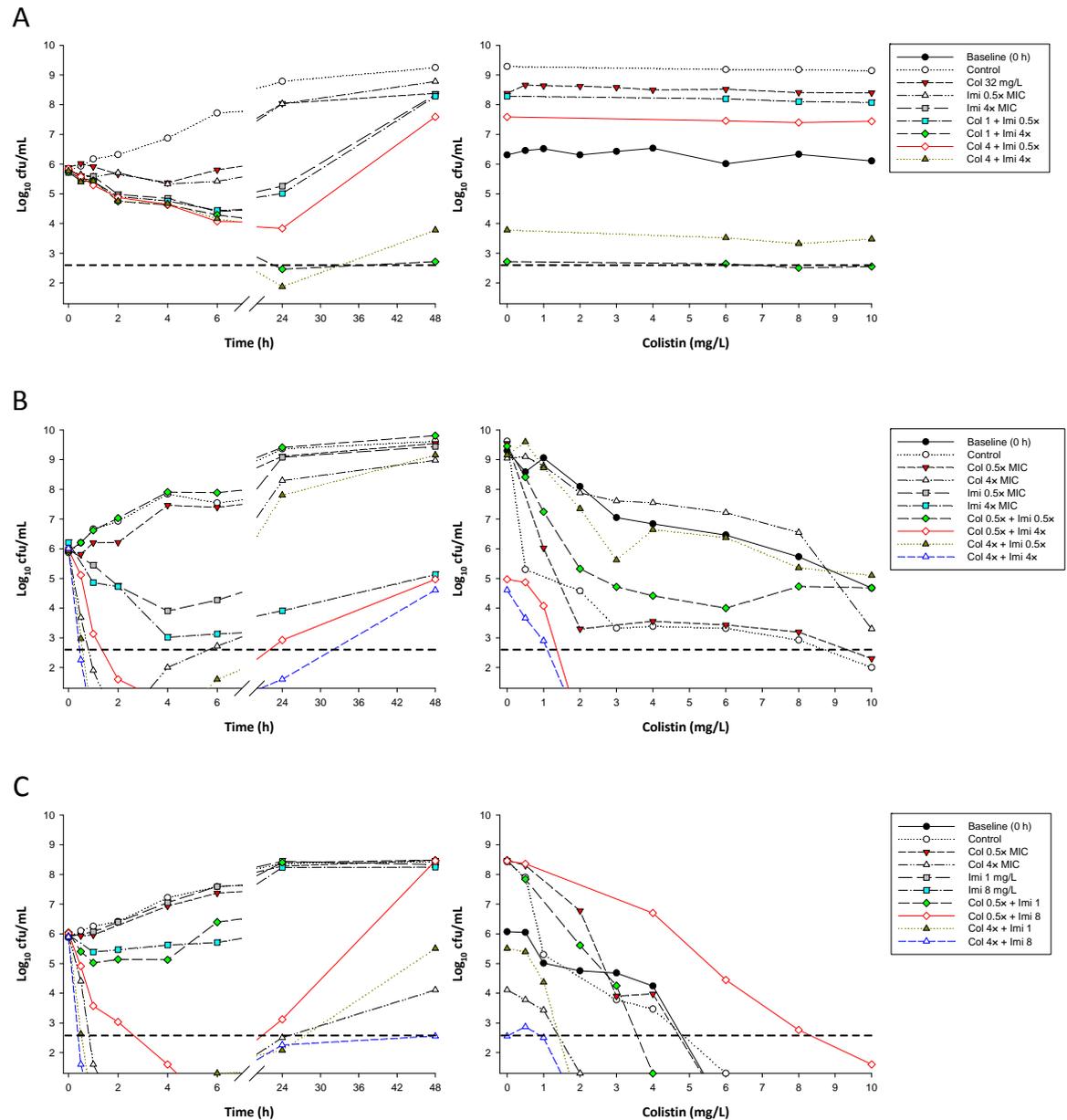


Figure 5-2. (Left) Representative time-kill curves with various clinically relevant concentrations of colistin (COL) and imipenem (IMI) alone and in combination at an inoculum of $\sim 10^6$ cfu/mL. (Right) PAPs at baseline (0 h) and after 48 h of exposure to colistin monotherapy, colistin-imipenem combination therapy, or neither antibiotic (control). (A) 19147 n/m (colistin resistant, imipenem susceptible, MDR); (B) 20509 n/m (susceptible to colistin and imipenem, non-MDR); (C) 20891 n/m (colistin susceptible, imipenem resistant, MDR). The Y-axis starts from the limit of detection, and the LOQ is indicated by the dashed horizontal line.

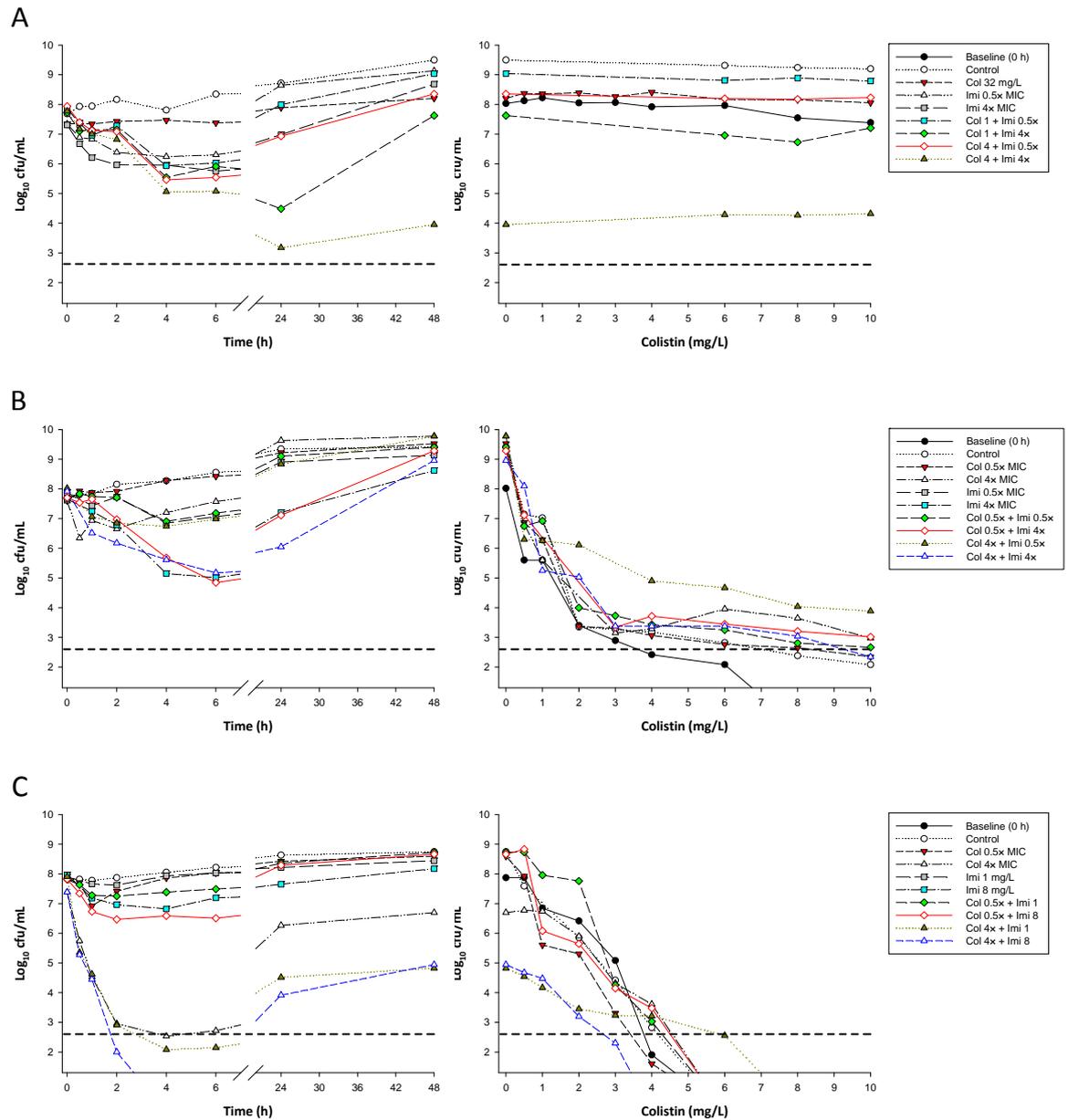


Figure 5-3. (Left) Representative time-kill curves with various clinically relevant concentrations of colistin (COL) and imipenem (IMI) alone and in combination at an inoculum of $\sim 10^8$ cfu/mL. (Right) PAPs at baseline (0 h) and after 48 h of exposure to colistin monotherapy, colistin-imipenem combination therapy, or neither antibiotic (control). (A) 19147 n/m (colistin resistant, imipenem susceptible, MDR); (B) 20509 n/m (susceptible to colistin and imipenem, non-MDR); (C) 20891 n/m (colistin susceptible, imipenem resistant, MDR). The Y-axis starts from the limit of detection, and the LOQ is indicated by the dashed horizontal line.

Table 5-2. Log changes in viable cell counts at 6, 24, and 48 h with various clinically relevant concentrations of colistin and imipenem against three *P. aeruginosa* isolates susceptible to both antibiotics^a

Isolate	Inoculum (cfu/mL)	Time (h)	Log change [$\log_{10}(\text{CFU}_t) - \log_{10}(\text{CFU}_0)$]										
			Col 0.5× MIC	Col 4× MIC	Imi 0.5× MIC	Imi 4× MIC	Imi 16× MIC	Col 0.5× MIC + Imi 0.5× MIC	Col 0.5× MIC + Imi 4× MIC	Col 0.5× MIC + Imi 16× MIC	Col 4× MIC + Imi 0.5× MIC	Col 4× MIC + Imi 4× MIC	Col 4× MIC + Imi 16× MIC
ATCC 27853	~10 ⁶	6	-0.41	-5.93	-0.03	-2.77	-2.83	-2.90	-4.39	-5.95	-4.69	-5.95	-5.99
		24	+3.20	+0.06	+2.91	-3.14	-3.66	+1.71	-3.58	-5.95	-2.20	-2.68	-3.34
		48	+3.80	+1.35	+3.60	-1.06	-1.81	+3.49	-2.39	-2.90	+0.04	-1.82	-2.27
19056 muc	~10 ⁸	6	+0.33	-2.48	-0.06	-2.04	-2.08	-1.00	-2.79	-2.73	-5.62	-4.94	-4.69
		24	+1.55	+0.05	+1.67	+0.14	-3.73	+1.50	-0.48	-3.42	-0.01	-3.51	-4.54
		48	+2.05	+1.65	+2.02	+1.96	-2.89	+2.04	+1.79	-3.30	+0.92	-2.25	-3.60
19056 muc	~10 ⁶	6	-2.34	-5.19	+0.45	-3.81	-5.49	-5.66	-5.69	-5.79	-5.88	-5.92	-5.75
		24	+1.63	-2.64	+2.66	-3.41	-5.49	+1.69	-4.39	-5.79	-2.62	-5.92	-5.75
		48	+3.13	+0.08	+3.27	+1.93	-5.49	+2.53	-0.27	-5.79	-0.44	-1.06	-5.75
	~10 ⁸	6	+0.11	-7.51	-0.83	-3.76	-4.22	-1.68	-4.00	-4.05	-7.96	-8.22	-7.95
		24	+0.79	-3.49	-0.01	-3.22	-5.02	+0.19	-3.46	-4.60	-2.50	-6.92	-7.95
		48	+1.42	-0.15	+0.47	+0.24	-6.08	+0.39	+0.27	-5.91	+0.08	-3.18	-7.95

Table 5-2. (Continued)

Isolate	Inoculum (cfu/mL)	Time (h)	Log change [$\log_{10}(\text{CFU}_t) - \log_{10}(\text{CFU}_0)$]										
			Col 0.5× MIC	Col 4× MIC	Imi 0.5× MIC	Imi 4× MIC	Imi 16× MIC	Col 0.5× MIC + Imi 0.5× MIC	Col 0.5× MIC + Imi 4× MIC	Col 0.5× MIC + Imi 16× MIC	Col 4× MIC + Imi 0.5× MIC	Col 4× MIC + Imi 4× MIC	Col 4× MIC + Imi 16× MIC
20509 n/m	~10 ⁶	6	+1.47	-3.18	-1.71	-3.08	-3.83	-2.29	-5.97	-6.14	-4.34	-6.01	-5.90
		24	+3.18	+2.39	+3.10	-2.30	-3.33	+3.26	-3.04	-3.93	+1.86	-4.41	-4.30
		48	+3.16	+3.07	+3.46	-1.08	-1.43	+3.48	-1.00	-1.68	+3.21	-1.40	-1.93
	~10 ⁸	6	+0.82	-0.01	-0.81	-2.65	-2.65	-0.52	-2.86	-2.55	-1.03	-2.75	-2.53
		24	+1.62	+2.04	+1.02	-0.45	-1.48	+1.40	-0.59	-2.60	+0.81	-1.88	-3.63
		48	+1.92	+2.19	+1.25	+0.96	-1.28	+1.70	+1.58	-0.98	+1.75	+1.03	-2.37

^a Col, colistin; Imi, imipenem. A gray background indicates activity (a reduction of $\geq 1 \log_{10}$ cfu/mL below the initial inoculum); a green background indicates synergy (a $\geq 2\text{-}\log_{10}$ decrease in the number of cfu/mL with the combination from that with its most active component); and a red background indicates additivity (a 1.0- to $<2\text{-}\log_{10}$ decrease in the number of cfu/mL with the combination from that with its most active component).

Table 5-3. Log changes in viable cell counts at 6, 24, and 48 h with various clinically relevant concentrations of colistin and imipenem against one colistin-resistant, imipenem-susceptible isolate and two colistin-susceptible, imipenem-resistant isolates of *P. aeruginosa*^a

Isolate	Inoculum (cfu/mL)	Time (h)	Log change [$\log_{10}(\text{CFU}_t) - \log_{10}(\text{CFU}_0)$]									
			Col 32 mg/L	Imi 0.5× MIC	Imi 4× MIC	Imi 16× MIC	Col 1.0 mg/L + Imi 0.5× MIC	Col 1.0 mg/L + Imi 4× MIC	Col 1.0 mg/L + Imi 16× MIC	Col 4.0 mg/L + Imi 0.5× MIC	Col 4.0 mg/L + Imi 4× MIC	Col 4.0 mg/L + Imi 16× MIC
Col resistant, Imi susceptible	~10 ⁶	6	-0.08	-0.35	-1.44	-1.28	-1.28	-1.46	-1.77	-1.77	-1.60	-1.83
		24	+2.16	+2.27	-0.58	-2.57	-0.71	-3.28	-4.57	-2.01	-3.89	-4.22
		48	+2.49	+3.02	+2.50	-4.55	+2.57	-3.03	-4.46	+1.74	-1.99	-3.54
	~10 ⁸	6	+0.04	-1.04	-1.56	-1.36	-1.83	-1.82	-1.85	-2.40	-2.70	-2.54
		24	+0.55	+1.31	-0.33	-2.67	+0.14	-3.24	-3.38	-1.10	-4.60	-4.61
		48	+0.86	+1.79	+1.37	-3.21	+1.18	-0.10	-3.19	+0.42	-3.82	-7.69

Table 5-3. (Continued)

Col susceptible, Imi resistant			Col	Col	Imi	Imi	Imi	Col	Col	Col	Col	Col	Col	
			0.5× MIC	4× MIC	1.0 mg/L	8.0 mg/L	32 mg/L	0.5× MIC + Imi 1.0 mg/L	0.5× MIC + Imi 8.0 mg/L	0.5× MIC + Imi 32 mg/L	0.5× MIC + Imi 1.0 mg/L	0.5× MIC + Imi 8.0 mg/L	0.5× MIC + Imi 32 mg/L	0.5× MIC + Imi 1.0 mg/L
19271 n/m	~10 ⁶	6	-1.89	-3.32	+1.68	+1.17	-0.54	-1.32	-2.00	-4.45	-3.43	-5.77	-5.71	
		24	+0.49	-2.19	+2.95	+2.89	-1.27	+0.71	+0.24	-5.75	-3.00	-5.77	-5.71	
		48	+2.87	+1.83	+3.01	+2.93	+1.56	+2.88	+2.81	-2.61	+0.15	-1.52	-5.71	
	~10 ⁸	6	-0.05	-2.22	+0.56	+0.26	-0.95	-1.05	-0.94	-1.57	-3.13	-3.48	-4.90	
		24	+0.67	-1.13	+0.84	+0.39	-1.70	-0.37	-0.08	-2.47	-1.86	-1.76	-3.88	
		48	+0.83	-1.26	+0.83	+0.46	+0.15	+0.94	+0.98	+0.25	-1.09	+0.22	-2.30	
	20891 n/m	~10 ⁶	6	+1.38	-5.95	+1.68	-0.26	-2.61	+0.35	-6.02	-5.90	-4.62	-5.90	-5.83
			24	+2.28	-3.43	+2.53	+2.27	-3.82	+2.35	-2.90	-5.90	-3.85	-3.64	-5.83
			48	+2.48	-1.84	+2.42	+2.28	+0.01	+2.43	+2.44	-3.82	-0.42	-3.34	-5.83
~10 ⁸		6	+0.21	-4.67	+0.13	-0.77	-2.50	-0.33	-1.32	-2.54	-5.76	-7.39	-7.52	
		24	+0.60	-1.12	+0.32	-0.31	-3.64	+0.54	+0.47	-2.38	-3.39	-3.47	-4.27	
		48	+0.77	-0.69	+0.55	+0.21	+0.07	+0.91	+0.84	+1.00	-3.08	-2.44	-2.89	

^a Col, colistin; Imi, imipenem. A gray background indicates activity (a reduction of ≥ 1 log₁₀ cfu/mL below the initial inoculum); a green background indicates synergy (a ≥ 2 -log₁₀ decrease in the number of cfu/mL with the combination from that with its most active component); and a red background indicates additivity (a 1.0- to < 2 -log₁₀ decrease in the number of cfu/mL with the combination from that with its most active component). For colistin-resistant isolate 19147 n/m, synergy or additivity was compared with imipenem monotherapy only.

Isolates susceptible to both colistin and imipenem. At the 10^6 -cfu/mL inoculum, the addition of colistin at $0.5\times$ MIC to imipenem (all concentrations) resulted in additivity or synergy at 6 h in 7 of 9 cases (i.e., 3 combinations against 3 isolates), achieving a ~ 2 - to 3-log_{10} greater kill than that with the most active equivalent monotherapy, and undetectable bacterial counts in many cases (Table 5-2 and Figure 5-2). By 24 or 48 h, improvements in activity with combination therapy over that with the most active monotherapy (usually imipenem) were modest, particularly when only clinically relevant concentrations of colistin ($0.5\times$ or $4\times$ MIC) were considered. Of the 27 cases (i.e., 9 combinations against 3 isolates), 7 at 24 h and 8 at 48 h showed additivity or synergy, although only 1 case resulted in activity (i.e., $\geq 1\text{-log}_{10}$ kill) if equivalent monotherapy with either drug was inactive. A similar pattern of activity was observed at the 10^8 -cfu/mL inoculum. For ATCC 27853, combinations containing colistin at $4\times$ MIC provided an additional $\sim 2\text{-log}_{10}$ kill to already active monotherapy at 6 h. Against all three isolates, there were 10 and 9 cases of additivity/synergy at 24 and 48 h, respectively, mostly involving colistin at 4 or $16\times$ MIC (Table 5-2).

Imipenem-resistant isolates. For the two imipenem-resistant isolates (19271 n/m and 20891 n/m), there was no evidence of carbapenemase activity; most likely, an alternative resistance mechanism, such as the loss of major outer membrane proteins, was present. At the low inoculum, combination therapy resulted in substantial improvements in bacterial kill with all colistin concentrations across 48 h. At 6 h, additivity/synergy occurred in 9 of 18 cases (i.e., 9 combinations across 2 isolates), predominantly against isolate 19271 n/m; additivity/synergy occurred with combinations containing colistin at all concentrations and produced additional reductions of ~ 2 - to 6-log_{10} cfu/mL over that with usually active colistin monotherapy (Table 5-3 and Figure 5-2). In 5 of 6 cases involving colistin at $4\times$ or $16\times$ MIC against 19271 n/m, bacterial counts were reduced to below the limit of detection (i.e., 20 cfu/mL). Substantial improvements in activity against both isolates were also observed at 24 and 48 h at all colistin concentrations.

Additivity/synergy occurred in 11 and 12 of 18 cases at 24 and 48 h, respectively, resulting in an additional ~1- to 4- \log_{10} kill at 24 h and >2.5- \log_{10} kill at 48 h over that with monotherapy (Table 5-3 and Figure 5-2). Interestingly, the combinations of colistin at 0.5 \times , 4 \times , or 16 \times MIC with imipenem at 32 mg/L each reduced the bacterial loads of both isolates to below the limit of detection at 24 h; the maximum reduction in bacterial counts (\log_{10} cfu/mL) at 24 h with colistin monotherapy at 16 \times MIC was ~4.5. Improvements in activity with combination therapy at the high inoculum also occurred at all time points but were essentially restricted to combinations containing colistin at 4 \times or 16 \times MIC. Ten of 12 cases at 6 h containing colistin 4 (Table 5-3) or 16 \times MIC (Appendix 2, Table A2-2) showed additivity or synergy. At 24 and 48 h, the addition of imipenem at all concentrations to colistin at 4 \times or 16 \times MIC produced additivity/synergy in more than half of all cases and substantially improved the activity over that with each antibiotic alone (by as much as ~4- \log_{10} kill).

Colistin-resistant isolate. Bacterial killing at the 10^6 -cfu/mL inoculum was substantially enhanced at 24 h; all combinations tested were additive or synergistic, and only one combination (colistin at 1 mg/L plus imipenem at 0.5 \times MIC) was inactive (Table 5-3). The addition of all colistin concentrations to imipenem at 4 \times or 16 \times MIC produced ~3.5 to 4.5- \log_{10} kill at 24 h, substantially higher than that with equivalent imipenem monotherapy. At 48 h, all colistin concentrations in combination with imipenem at 4 \times MIC were synergistic (~2- to 4- \log_{10} kill) and substantially improved activity over that with equivalent monotherapy. The addition of colistin at 32 mg/L to imipenem (all concentrations) was additive or synergistic at a substantially earlier time (6 h), with ~1- to 2- \log_{10} greater kill than with the equivalent imipenem monotherapy (overall kill, ~3- \log_{10} cfu/mL). At the high inoculum, additivity was achieved at 6 h with all combinations containing colistin at 4 mg/L (Table 5-3) and 32 mg/L (Appendix 2, Table A2-2), and activity was enhanced by ~1- \log_{10} kill over that with imipenem monotherapy. Eight of 9 combinations at 24 h and 7 of 9 combinations at 48 h were additive or synergistic, encompassing all colistin

concentrations and in many cases resulting in additional reductions of ~1- to 4- \log_{10} cfu/mL over that with the most active monotherapy (imipenem at 16 \times MIC). This enhancement of activity was particularly evident with combinations containing colistin at 4 or 32 mg/L, and on two occasions, when colistin was combined with imipenem at 16 \times MIC, no viable bacteria were detected at 48 h.

5.4.2 Emergence of colistin resistance

For the 4 colistin-heteroresistant isolates (Table 5-1), the proportion of resistant subpopulations at 10^8 cfu/mL ranged from 2.2×10^{-7} to 4.7×10^{-3} (Figure 5-1). With colistin monotherapy against the isolates susceptible to both colistin and imipenem, real-time PAPs performed at 48 h in the time-kill studies demonstrated increases in colistin-resistant subpopulations at both the low and high inocula with clinically relevant colistin concentrations (examples are shown in Figures 5-2 and 5-3 and Appendix 2); no such increase was observed with isolate 19056 muc at the high inoculum. Against imipenem-resistant isolate 19271 n/m, colistin concentrations of 0.25 \times to 64 \times MIC at the low inoculum and 1 \times to 64 \times MIC at the high inoculum resulted in nearly 100% of the remaining cells at 48 h growing in the presence of 10 mg/L colistin. In contrast, no increase in colistin-resistant subpopulations was observed for the imipenem-resistant isolate 20891 n/m at either inoculum. Combination therapy against colistin-susceptible isolates generally had little effect on the proportion of colistin-resistant subpopulations at 48 h at either inoculum; the shapes of the PAPs were very similar to that obtained with equivalent colistin monotherapy (Figures 5-2 and 5-3).

5.5 Discussion

Although colistin has been commercially available for more than 50 years (39), reliable PK/PD data have emerged only recently. Population PK studies have shown that plasma colistin concentrations achieved with currently recommended CMS dosage regimens are likely to be

suboptimal for many patients, typically generating average steady-state plasma colistin concentrations of ~2 to 3 mg/L, with some patients achieving concentrations as high as ~10 mg/L (91-95, 97). Increasing the daily dose of CMS for such patients may not be an option, since nephrotoxicity, which occurs in ~30 to 50% of patients (224, 235), is a dose-limiting adverse effect. Given these circumstances and the current last-line status of colistin therapy, we chose to examine not only synergy but also additivity, since even a relatively small increase in activity with combination therapy may be beneficial for patient care. Because colistin is almost entirely unbound in CAMHB (528), colistin concentrations of 0.5× and 4× MIC for isolates with MICs ≤1 mg/L and 16× MIC for isolates with MICs of ≤0.5 mg/L (1 and 4 mg/L for colistin-resistant isolates) used in our study are clinically relevant, even assuming that binding of colistin by plasma in patients is similar to that in animals (i.e., ~50% bound) (417). Considering the effect of protein binding, all the imipenem concentrations employed are readily achieved in plasma (534).

Because some data show that activities of both colistin (385) and imipenem (535) are attenuated at high inocula compared to those at low inocula, experiments were conducted at inocula of both ~10⁶ and ~10⁸ cfu/mL. An inoculum effect was generally observed for colistin monotherapy, whereas no obvious inoculum effect was present for imipenem (Figures 5-2 and 5-3). Regrowth of all isolates was observed with colistin monotherapy, even at colistin concentrations well above those that can be safely achieved clinically. Similar regrowth with colistin (or polymyxin B) monotherapy has been observed for colistin-susceptible *P. aeruginosa* both *in vitro* (74, 385, 387, 517) and *in vivo* (395). For *A. baumannii* and *K. pneumoniae*, regrowth following colistin monotherapy has been attributed to the amplification of colistin-resistant subpopulations (126-127, 394), with colistin heteroresistance reported in both species (124, 127, 529-530). We have reported here, for the first time, colistin heteroresistance in *P. aeruginosa*. The emergence of colistin resistance following colistin monotherapy has been reported previously for *P. aeruginosa*

at both low and high inocula (385, 517), and a similar phenomenon was observed in the present study for all isolates except 20981 n/m. While *P. aeruginosa* can undergo adaptive resistance to polymyxins (331), the presence of colistin heteroresistance at baseline and the changes in PAPs after treatment suggest regrowth following colistin monotherapy may be due to amplification of preexisting colistin-resistant subpopulations. This possibility suggests that care is required with colistin monotherapy against *P. aeruginosa*, even where isolates appear susceptible on the basis of MICs.

The addition of imipenem to colistin at both inocula generally resulted in substantial improvements in bacterial killing over that with equivalent monotherapy against MDR *P. aeruginosa* isolates resistant to either antibiotic, even when ESBLs were present. The improvements in activity against these isolates were observed across the 48-h duration, with all colistin concentrations at the low inoculum and with colistin at 4× and 16× MIC (or 4 and 32 mg/L) at the high inoculum. Notably, the total reductions in bacterial counts (\log_{10} cfu/mL) achieved with combinations containing lower colistin concentrations (0.5× and 4× MIC or 1 and 4 mg/L) were on many occasions similar in magnitude to the reductions achieved with combinations containing colistin at 16× MIC, particularly at the 10^6 -cfu/mL inoculum (Table 5-3). This suggests that combinations of colistin and imipenem containing clinically relevant colistin concentrations may be as effective as combinations containing higher concentrations against MDR isolates when resistance to either drug is present. This is an important result, given that colistin-induced nephrotoxicity is a dose-limiting adverse effect.

The benefits of the addition of imipenem to colistin for overall antibacterial activity were less pronounced against the three isolates susceptible to both antibiotics and were generally restricted to improvements in initial kill, i.e. up to 6 h (Table 5-2). Because a proportion of patients will achieve only low plasma colistin concentrations with the currently recommended

dosage regimens (95, 97), the combination of colistin and imipenem at the commencement of therapy may help to quickly reduce bacterial levels so as to facilitate clearance by the immune system.

Previous time-kill studies have examined colistin in combination with carbapenems against *P. aeruginosa* (410, 460, 476, 481, 536). These studies examined colistin with imipenem, meropenem, or doripenem at a single inoculum ($\sim 10^6$ or $\sim 10^7$ cfu/mL), though the emergence of colistin resistance was not examined (e.g., by the use of PAPs). The present study is the first to investigate the emergence of colistin resistance with colistin combination therapy. In the present investigations, in cases where the combination led to extensive killing at 48 h, meaningful interpretation of the PAPs was not possible (e.g., Figure 5-2B, colistin at 4× MIC as monotherapy and in combination with imipenem at 4× MIC). When bacterial numbers at 48 h were comparable, changes in PAPs with combination therapy generally mirrored those observed with equivalent exposure to colistin as monotherapy. However, in both the present study and previously reported studies (410, 460, 476, 481, 536), static concentrations and the instability of carbapenems in aqueous media may have contributed to the regrowth and the emergence of colistin resistance at 48 h (537). Thus, it will be important to further assess the utility of these combinations against a range of isolates with varying susceptibilities (including heteroresistant strains) in dynamic *in vitro* models and *in vivo*.

Two possible reasons for the enhanced pharmacodynamic effect observed with the combination of colistin and imipenem are subpopulation synergy and mechanistic synergy, as proposed previously (452). Subpopulation synergy involves one drug killing the subpopulation(s) resistant to the other drug, and *vice versa*. Four of the six isolates in the present study were colistin heteroresistant (Table 5-1), indicating the existence of colistin-resistant subpopulations prior to therapy. In addition, the four imipenem-susceptible isolates were imipenem heteroresistant

(while the MIC was ≤ 4 mg/L, subpopulations grew in the presence of >4 mg/L imipenem [data not shown]). Another possibility is mechanistic synergy, whereby colistin and imipenem acting on different cellular pathways, each increase the rate or extent of killing of the other drug. In Gram-negative bacteria, carbapenems must first gain entry into the periplasmic space in order to bind to critical penicillin-binding proteins located on the cytoplasmic membrane (538-539). A number of resistance mechanisms may operate to limit the concentration of carbapenems in the periplasm, including the presence of carbapenem-hydrolysing enzymes and loss of outer membrane proteins (539). Polymyxins cause considerable permeabilisation of the outer membrane (291). It is possible that the effect of colistin on membrane permeability results in substantially increased concentrations of imipenem in the periplasm and improved bactericidal activity. Subpopulation and mechanistic synergy are not mutually exclusive; both may operate simultaneously. Further studies, including mechanism-based mathematical modeling, to investigate the mechanism(s) underpinning the enhanced pharmacodynamic activity observed are ongoing.

In the battle against rapidly emerging bacterial resistance in Gram-negative ‘superbugs’, rational approaches to the use of combinations of existing antibiotics may be greatly beneficial. To the best of our knowledge, this is the first systematic study on the PD of colistin in combination with imipenem against *P. aeruginosa*, including MDR and colistin heteroresistant strains, at both low and high inocula. Clinically relevant concentrations of colistin in combination with imipenem substantially increased bacterial killing against MDR *P. aeruginosa* isolates at both inocula when isolates were resistant to either antibiotic. Further investigations in *in vitro* pharmacodynamic systems, animal infection models, and clinical studies are warranted to optimize colistin-imipenem combinations targeting both colistin-susceptible and colistin-resistant subpopulations.

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The content of this paper 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.

Declaration for Thesis Chapter 6

Declaration by candidate

In the case of Chapter 6, the nature of my contribution to this manuscript included the following:

- design of the study;
- all laboratory experiments;
- data analysis and interpretation;
- preparation of the initial draft of the manuscript and subsequent revisions; and
- formulation of the conclusions and hypothesis arising from the results of the study.

The extent of my contribution was 80%. The following co-authors contributed to the work:

Name	Nature of contribution
Brian T. Tsuji	<ul style="list-style-type: none">• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;• review of manuscript drafts and revisions; and• formulation of conclusions and hypotheses arising from the results of the study.
Jurgen B. Bulitta	<ul style="list-style-type: none">• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;• review of manuscript drafts and revisions; and• formulation of conclusions and hypotheses arising from the results of the study.
Alan Forrest	<ul style="list-style-type: none">• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;• review of manuscript drafts and revisions; and• formulation of conclusions and hypotheses arising from the results of the study.
Jovan Jacob	<ul style="list-style-type: none">• performed assays for colistin and CMS concentrations; and• review of manuscript drafts and revisions
Hanna Sidjabat	<ul style="list-style-type: none">• performed PCR assays; and• review of manuscript drafts and revisions
David L. Paterson	<ul style="list-style-type: none">• interpretation of PCR results; and• review of manuscript drafts and revisions

Roger L. Nation	<ul style="list-style-type: none"> • supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation; • review of manuscript drafts and revisions; and • formulation of conclusions and hypotheses arising from the results of the study.
Jian Li	<ul style="list-style-type: none"> • supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation; • review of manuscript drafts and revisions; and • formulation of conclusions and hypotheses arising from the results of the study.

Candidate's signature	Date
Phillip J. Bergen	

Declaration by co-authors

The undersigned hereby certify that:

1. the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors;
2. they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
3. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
4. there are no other authors of the publication according to these criteria;
5. potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
6. the original data are stored at the Facility for Anti-infective Drug Development and Innovation (FADDI; Monash Institute of Pharmaceutical Sciences), and will be held for at least five years from the date indicated below.

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Chapter Six

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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6.1 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 ($\sim 10^6$ and $\sim 10^8$ cfu/mL) against a colistin-heteroresistant reference strain (ATCC 27853) and colistin-resistant, MDR clinical isolate (19147 n/m). Four combinations utilising 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 at 2 mg/L increased activity against ATCC 27853 at the high inoculum; only colistin at 0.5 mg/L plus doripenem at 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 at 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 optimisation of colistin/doripenem combinations.

6.2 Introduction

Multidrug-resistant *Pseudomonas aeruginosa* is one of several important Gram-negative bacteria emerging as significant pathogens worldwide (10, 13). With a very limited number of therapeutic options remaining against these pathogens, and a lack of novel antimicrobial agents in the drug development pipeline (13, 58), particularly those with activity against *P. aeruginosa* (13), clinicians have been forced to reexamine the use of 'old', previously discarded drugs such as the polymyxins (10, 154). 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 (38-39). 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 (39, 45, 524). Several institutions have already experienced outbreaks of multidrug-resistant (MDR) Gram-negative bacteria resistant to all commercially available antibiotics except the polymyxins (60, 171, 497). Of particular concern is that with the rapid increase in use of colistin over the last decade, especially in critically-ill patients (10, 39), has come a concomitant increase in the number of reports of resistance to colistin (39, 45, 62).

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 (95, 97). In addition, both *in vitro* (126-127, 517, 528) and *in vivo* (395, 527) 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* (116, 529), *Klebsiella pneumoniae* (127, 530), and most recently in *P. aeruginosa* (540). 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.

6.3 Materials and Methods

6.3.1 Bacterial isolates

Two strains of *P. aeruginosa* were employed in this study: a colistin-heteroresistant reference strain, ATCC 27853 (American Type Culture Collection, Manassas, VA), 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 the existence, in an isolate for which the colistin minimum inhibitory concentration (MIC) was ≤ 2 mg/L, of subpopulations 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 (29). 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, Ca²⁺ at 23.0 mg/L, Mg²⁺ at 12.2 mg/L; Oxoid, Hampshire, England) via broth microdilution (152). Resistance to colistin (152) and doripenem (541) was defined as MIC \geq 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 -80°C in cryovials (Simport Plastics, Boloel, Quebec, Canada).

6.3.2 Antibiotics and reagents

For MIC determinations and *in vitro* PK/PD studies, colistin sulphate (lot 109K1574, 23,251 units/mg) was purchased from Sigma-Aldrich, St Louis, MO, while doripenem (lot 0137Y01) was kindly donated by Johnson and Johnson, Shionogi and Co, Osaka, Japan. Colistin (sulfate) was employed in the current study as colistin is the active antibacterial agent formed *in vivo* after administration of its inactive prodrug, colistin methanesulfonate (CMS) (515). 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 minimise loss from degradation, then sterilised by filtration with a 0.22- μ m-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 (80).

6.3.3 Binding of doripenem in growth medium

The binding of doripenem in CAMHB was measured by equilibrium dialysis using Dianorm equilibrium dialyser 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 dialysed 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 analysed as described below. The fraction of doripenem unbound in CAMHB (f_u) was calculated as follows: (acceptor doripenem concentration)/(donor doripenem concentration).

6.3.4 *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 ($\sim 10^6$ and $\sim 10^8$ cfu/mL) using a one-compartment *in vitro* PK/PD model described previously (517) and below. Prior to each experiment, strains were subcultured onto horse blood agar (Media Preparation Unit, The University of Melbourne, Parkville, Australia) and incubated at 35°C for 24 h. One colony was then selected and grown overnight in 10 mL of CAMHB, from which early log-phase growth was obtained. For a starting inoculum of $\sim 10^6$ 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 $\sim 10^6$ cfu/mL. To achieve a starting inoculum of $\sim 10^8$ 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 37°C and a magnetic stir bar to ensure adequate mixing. One compartment acted as a control to define growth dynamics in the absence of antibiotic, while colistin and/or doripenem

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 6-1); 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 (95, 97). For colistin-containing regimens at the higher inoculum ($\sim 10^8$ 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 6-1); 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 (542).

Three constant concentration colistin and three intermittent doripenem dosage regimens were simulated for monotherapy (Table 6-1). 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 6-1);

combination dosage regimens mimicked the PK profiles of each drug achieved in critically-ill patients (94-95, 97). As we have previously demonstrated that colistin (528), and in the present study doripenem, are almost entirely unbound in CAMHB, the specified concentrations represent unbound (free) concentrations.

Table 6-1. Colistin (Col) and doripenem (Dor) dosage regimens, PK/PD index values and sampling times in the *in vitro* PK/PD model

	Dosage regimens at $\sim 10^6$ and $\sim 10^8$ cfu/mL starting inocula						Combination therapy
	Col monotherapy ^{a,b}			Dor monotherapy ^c			
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 ^d							
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 ^e	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

^a Colistin dosage regimens involved a constant concentration of colistin simulating continuous infusion.

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

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

^d 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.

^e cfu/mL determined at all times. Full PAPs were performed at 0 and 96 h; 'mini PAPs' were performed at 6, 24, 48 and 72 h.

6.3.5 Microbiological response and the emergence of resistance to colistin

Serial samples (0.6 mL) were collected aseptically at times shown in Table 6-1 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 spirally plating (WASP, Don Whitley Scientific, West Yorkshire, UK) 50 μ L of appropriately diluted sample (using 0.9% saline) onto either nutrient agar (viable counting) or Mueller-Hinton agar (PAPs), followed by incubation at 35°C 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 (94-95, 97). 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 (Synbiosis, Cambridge, 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.

6.3.6 Pharmacokinetic validation

Samples (100 μ L) collected in duplicate from the *in vitro* PK/PD experiments were placed in 1.5-mL microcentrifuge tubes (Greiner Bio-one) and immediately stored at -80°C until analysis; all samples were assayed within 4 weeks. Concentrations of colistin were measured using high-performance liquid chromatography (HPLC) (417) 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 μ L of sample, 100 μ L of 3-(N-morpholino)propanesulfonic acid (MOPS) buffer and 400 μ L of methanol were added, vortexed and centrifuged at 10,000 rpm for 10 min. An aliquot of the sample (50 μ L) was injected onto a Phenosphere-NEXT 5 μ C18 column (250 mm \times 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.

6.3.7 Pharmacodynamic analysis

Microbiological responses to monotherapy and combination therapy were examined using the log change method, comparing the change in bacterial counts (\log_{10} cfu/mL) from that at 0 h [$\log_{10}(\text{CFU}_0)$] to that at a given time (t) (6, 24, 48, 72, or 96 h) [$\log_{10}(\text{CFU}_t)$], as follows:

$$\text{log change} = \log_{10}(\text{CFU}_t) - \log_{10}(\text{CFU}_0)$$

Single antibiotic or combination regimens causing a reduction of ≥ 1 - \log_{10} cfu/mL from the initial inoculum at 6, 24, 48, 72, or 96 h were considered active. We considered synergy to be indicated by a ≥ 2 - \log_{10} cfu/mL-lower bacterial count with the combination than with its most active component at the specified time (464); additivity was defined by a 1 to < 2 - \log_{10} cfu/mL-lower bacterial count with the combination.

6.4 Results

6.4.1 Pharmacokinetic validation and doripenem binding

The colistin drug concentrations achieved (mean \pm 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 6-1. 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.

6.4.2 Microbiological response

The initial inocula (mean \pm 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 6-2 (ATCC 27853) and Figure 6-3 (19147 n/m). Log changes of viable cell counts at each inoculum with mono- and combination therapy are presented in Table 6-2.

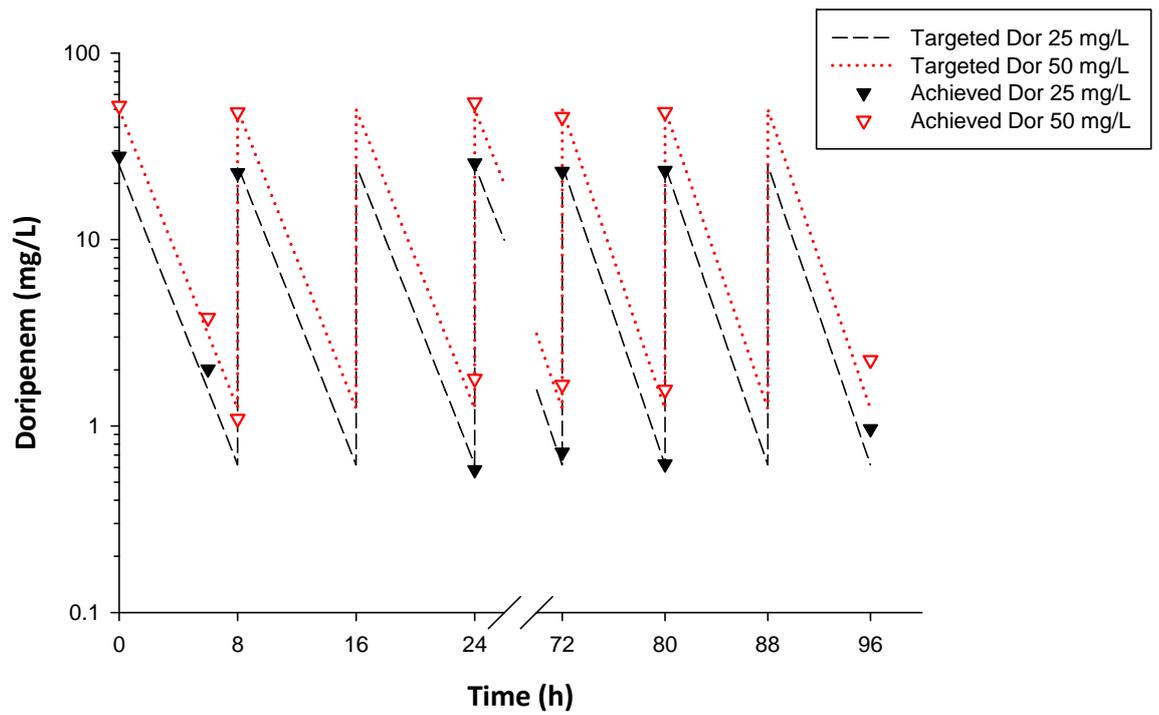


Figure 6-1. Targeted doripenem (Dor) pharmacokinetic profiles for 25 and 50 mg/L 8-hourly regimens with measured Dor concentrations.

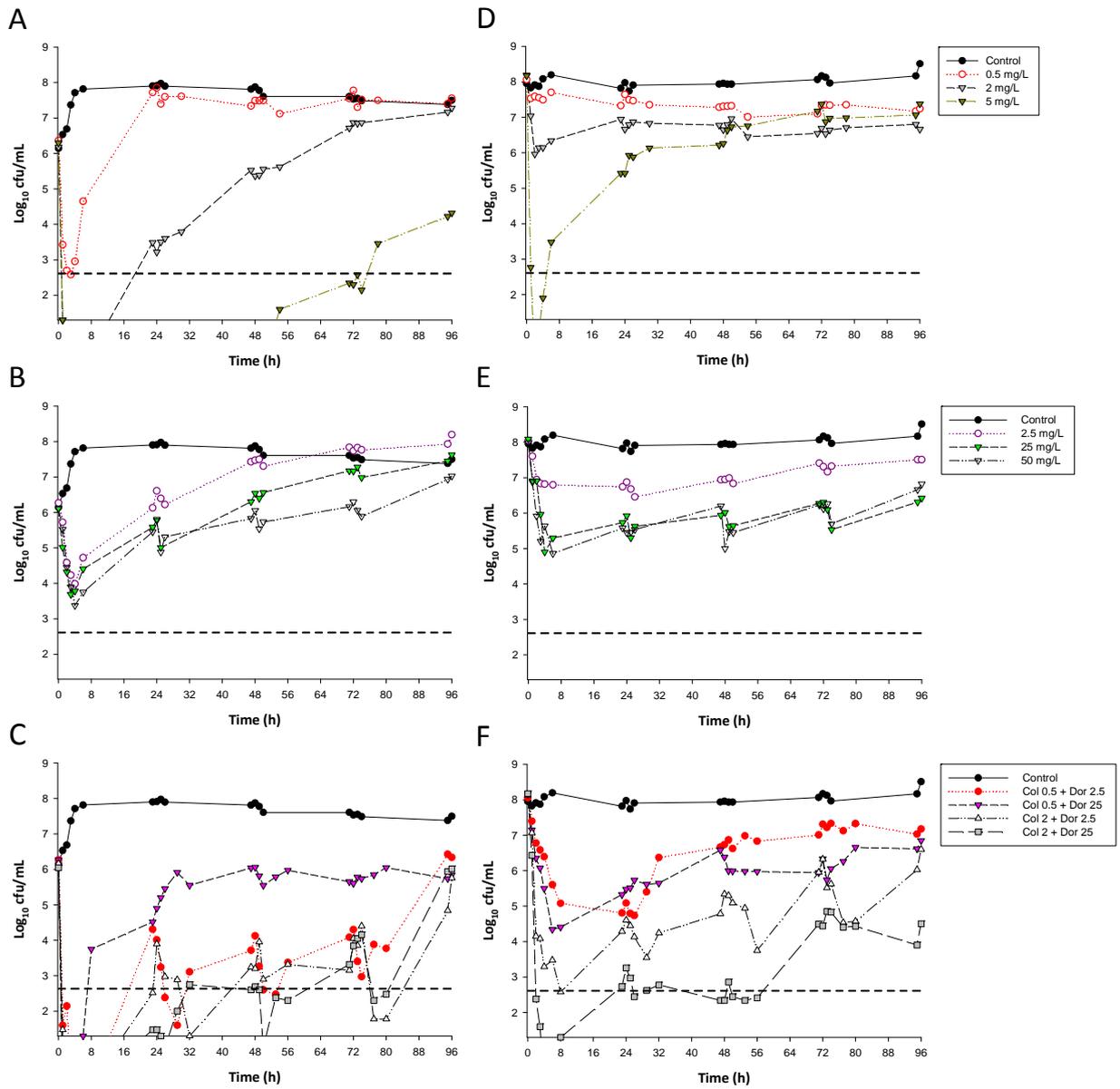


Figure 6-2. Time-kill curves for colistin monotherapy (Panels A and D), doripenem monotherapy (Panels B and E) and the combination (Panels C and F) against ATCC 27853 at the 10^6 cfu/mL (left-hand panels) and 10^8 cfu/mL (right-hand panels) inocula. The Y-axis starts from the limit of detection and the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

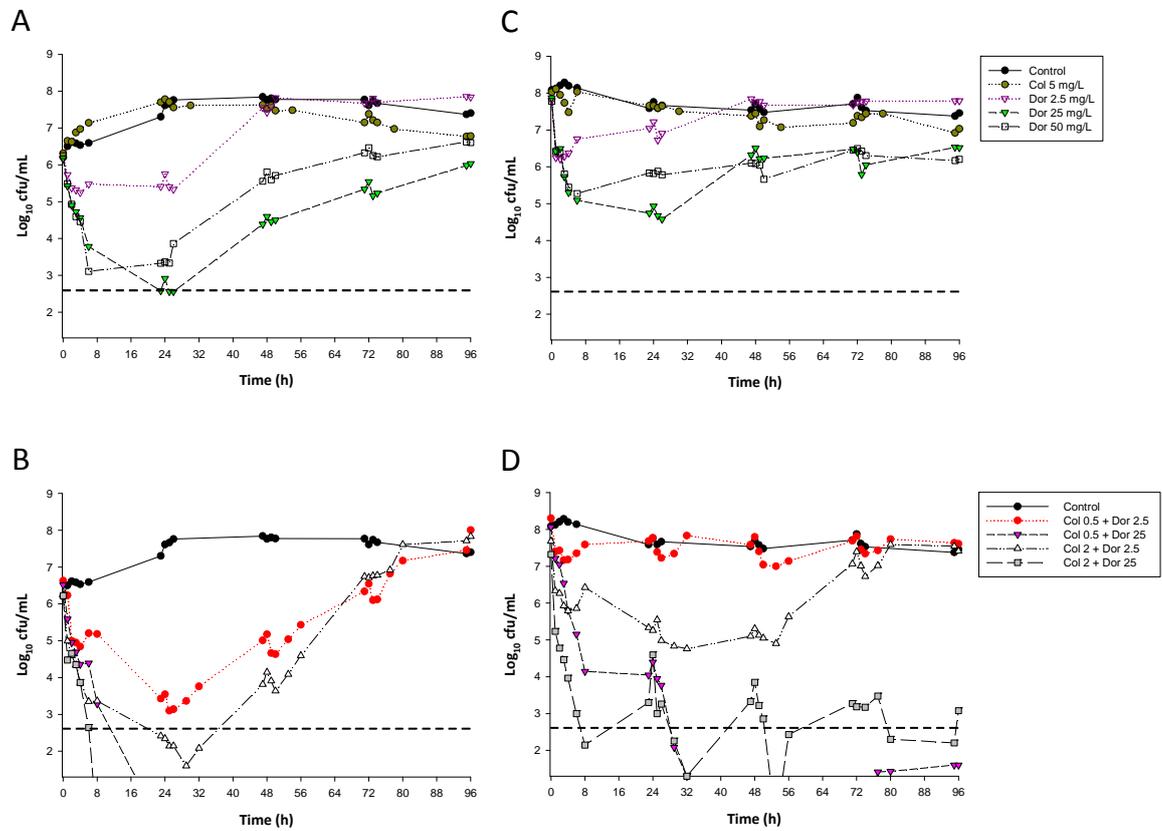


Figure 6-3. Time-kill curves for colistin and doripenem monotherapy (Panels A and C) and the combination (Panels B and D) against 19147 n/m at 10^6 cfu/mL (left-hand panels) and 10^8 cfu/mL (right-hand panels) inocula. The Y-axis starts from the limit of detection and the limit of quantification (LOQ)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

Table 6-2. Log changes in viable cell counts at 6, 24, 48, 72, and 96 h with colistin and/or doripenem against two *P. aeruginosa* isolates^a

Isolate	Inoculum (cfu/mL)	Time (h)	Log change [$\log_{10}(\text{CFU}_t) - \log_{10}(\text{CFU}_0)$]									
			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 ^b	~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

Table 6-2. (Continued)

Isolate	Inoculum (cfu/mL)	Time (h)	Log change [$\log_{10}(\text{CFU}_t) - \log_{10}(\text{CFU}_0)$]									
			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
19147 n/m ^c	~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

^a Col, colistin; Dor, doripenem. A gray background indicates activity (a reduction of $\geq 1 \log_{10}$ cfu/mL below the initial inoculum); a green background indicates synergy (a $\geq 2\text{-log}_{10}$ decrease in the number of cfu/mL with the combination from that with its most active component); and red background indicates additivity (a 1.0- to $< 2\text{-log}_{10}$ decrease in the number of cfu/mL with the combination from that with its most active component).

^b Colistin-heteroresistant reference strain; colistin heteroresistance was defined as the existence, in a colistin-susceptible isolate (i.e., MIC, ≤ 2 mg/L) of subpopulations that were able to grow in the presence of > 2 mg/L colistin.

^c Non-mucoid MDR, colistin-resistant clinical isolate; colistin monotherapy performed with 5 mg/L only.

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 (Figure 6-2A). 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 $\sim 4 \log_{10}$ 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 (Figure 6-2D). 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 $\sim 3.5 \log_{10}$ 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 (Figures 6-3A and 6-3C).

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 $\sim 2.5\text{-}\log_{10}$ cfu/mL, with regrowth beginning by 6 h (Figure 6-2B). 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 $\sim 0.5\text{-}$ to 1-log below control values, and 25 and 50 mg/L regimens bacterial counts $\sim 1.5\text{-}$ to 3-log below control values (Figure 6-2E). Against the MDR isolate, doripenem 2.5 mg/L 8-hourly produced only minimal bacterial killing ($\sim 1\text{-}$ to $2\text{-}\log_{10}$ kill) at each inoculum, with regrowth close to control values by 24 to 48 h (Figures 6-3A and 6-3C). Higher doripenem concentrations (25 and 50 mg/L) produced rapid initial killing of $\sim 3\text{-}\log$ at 6 h, with subsequent regrowth to within $\sim 1\text{-}\log$ of control values at 96 h (Figures 6-3A and 6-3C). 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 $\sim 2.5\text{-log}_{10}$ 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 6-2). Both combinations resulted in synergy or additivity at most time points across 96 h (Table 6-2). Synergy was particularly evident with the combination of colistin 0.5 mg/L and doripenem 2.5 mg/L, with $\sim 3\text{- to }4\text{-log}_{10}$ 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) to colistin 2 mg/L produced synergy at 48 and 72 h, and remained additive at 96 h with regrowth close to the level of the initial inoculum (Figure 6-2C and Table 6-2). At the high inoculum, combinations of colistin 0.5 mg/L and doripenem (2.5 or 25 mg/L) produced only modest increases in bacterial killing across the first 8 to 24 h, with regrowth thereafter similar to that of the most active single agent (doripenem) (Figure 6-2F). With combinations containing colistin 2 mg/L, rapid and substantial reductions in bacterial counts were observed with an additional $\sim 3.5\text{ log}_{10}$ cfu/mL kill over the most active monotherapy achieved at 8h with doripenem 2.5 mg/L, and an additional $\sim 5\text{ log}_{10}$ cfu/mL kill achieved at 4 h with doripenem 25 mg/L; with the latter combination, no viable bacteria were detected at this time. Synergy or additivity was maintained with these combinations across 48 and 96 h with doripenem 2.5 and 25 mg/L, respectively (Table 6-2).

Against 19147 n/m at the 10^6 inoculum, colistin 0.5 mg/L plus doripenem 2.5 mg/L produced synergy at 24 and 48 h, with regrowth approaching control values by 72 to 96 h (Figure 6-3B and Table 6-2). A similar killing profile was generated with the combination of colistin 2 mg/L and doripenem 2.5 mg/L, although initial bacterial killing was greater ($\sim 3\text{ log}$ kill) and lower bacterial counts maintained across the first ~ 60 h (Figure 6-3B). With this latter regimen, bacterial counts as low as 1.6 log_{10} cfu/mL (at 29 h) were observed. With combinations containing colistin (0.5 or 2 mg/L) and doripenem 25 mg/L, the initial rate and extent of killing up to 4 – 6 h was similar to

that of doripenem monotherapy (Figure 6-3B). By 8 and 24 h, no viable bacteria were observed with the combinations containing colistin 2 and 0.5 mg/L, respectively, and no regrowth was subsequently detected. At the high inoculum, the combination of colistin 0.5 mg/L and doripenem 2.5 mg/L was essentially inactive (Figure 6-3D). Increasing the concentration of colistin to 2 mg/L produced greater bacterial kill at both 24 h (additive) and 48 h (synergistic), with regrowth to control levels by 72 h (Figure 6-3D and Table 6-2). 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) (Figure 6-3D). 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 (Figure 6-3D).

6.4.3 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 6-3). Colistin 0.5 or 2 mg/L resulted in substantial increases in the proportion of colistin-resistant subpopulations at both inocula (Figure 6-4 and Table 6-3). 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 (Figure 6-3 and Table 6-3). 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).

Table 6-3. 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

* ND, No colistin-resistant subpopulations detected.

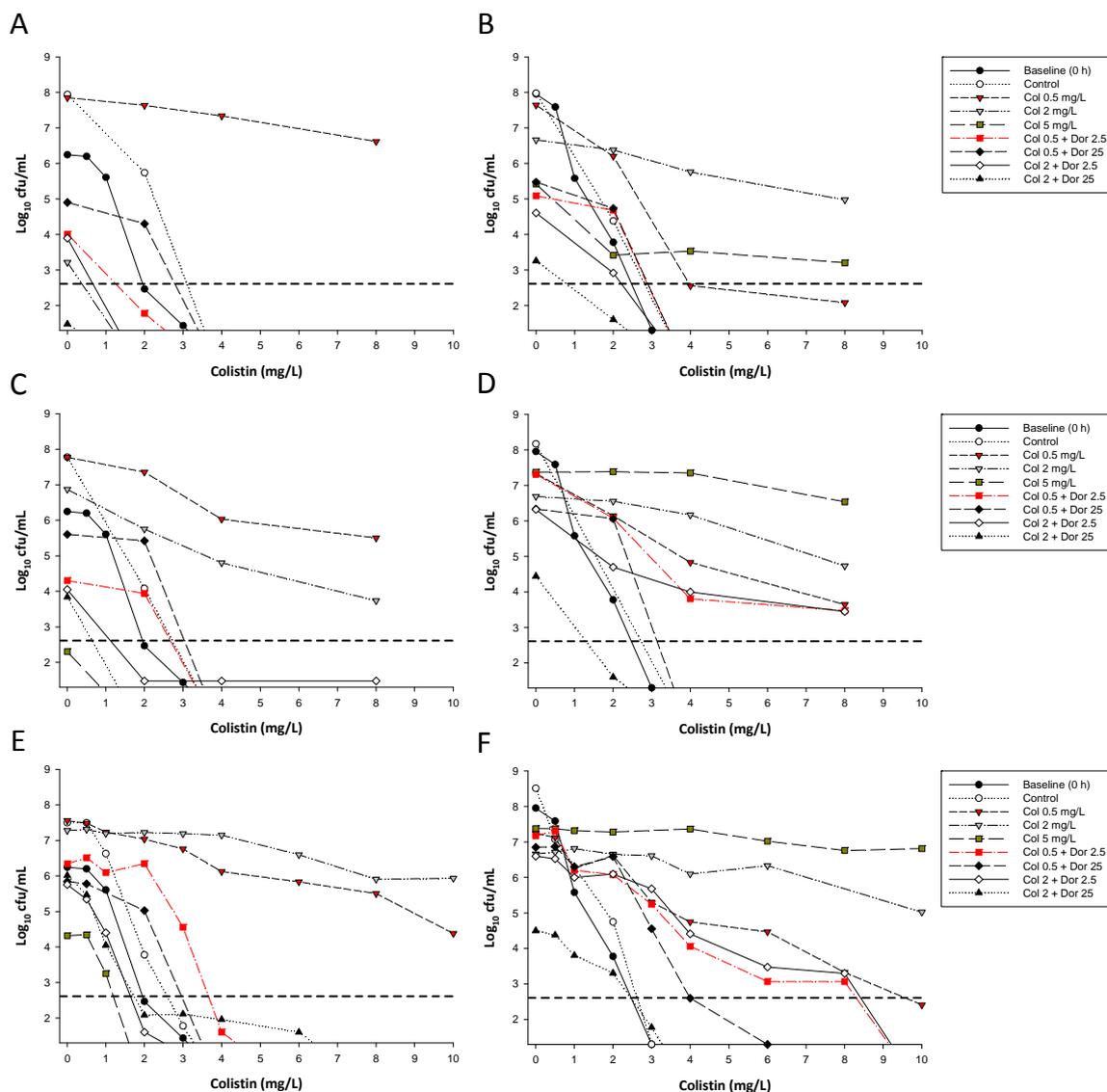


Figure 6-4. Population analysis profiles (PAPs) against ATCC 27853 with colistin monotherapy, colistin plus doripenem combination therapy or neither antibiotic (control) at 10^6 cfu/mL inoculum (left-hand panels) and 10^8 cfu/mL inoculum (right-hand panels), at 24 h (Panels A and B), 72 h (Panels C and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

Combination therapy against ATCC 27853 substantially reduced the emergence of colistin-resistant subpopulations (Table 6-3). 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 (Figure 6-4). 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) (Figure 6-4). 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 (Figure 6-4F). 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).

6.5 Discussion

Colistin is increasingly used as salvage therapy in critically-ill patients for otherwise untreatable MDR infections (38-39). However, regrowth of colistin-susceptible *P. aeruginosa* with colistin (or polymyxin B) monotherapy is commonly observed (74, 385, 387, 395, 517), 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 (95, 97). 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 (19). 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* (543-544) and its low

potential for selection of carbapenem-resistant *P. aeruginosa* (545-547). As some data show that activity of colistin (385) and carbapenems alone (535) is attenuated at high compared to low inocula, in the present study experiments were conducted at both $\sim 10^6$ and $\sim 10^8$ 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 $\sim 2 - 3$ mg/L, with some patients achieving concentrations up to ~ 10 mg/L (94-95, 97). As colistin concentrations at steady-state remain more or less constant (95, 97), colistin was administered as a constant infusion. We have previously demonstrated that colistin is almost entirely unbound in CAMHB (528). 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., $\sim 50\%$ bound) (417). 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 (548). Doripenem is typically administered intermittently every 8 h, with a standard 500 mg dose achieving a C_{max} of ~ 25 mg/L (541, 549). 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 (541, 549-551).

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 (Figures 6-2 and 6-3). 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 (410, 460, 476, 481, 540). 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 (540); 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 (331), the report of colistin heteroresistance in *P. aeruginosa* (540), and changes in PAPs following treatment with colistin monotherapy (385, 517, 540), 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 $\sim 1\text{-}2 \log_{10}$ 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 6-3). 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 (540). Loss of imipenem due to degradation in the static experiments likely contributed to this result (537). 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 (452). 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 permeabilisation of the outer membrane by colistin (291) 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 (538-539). 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 optimisation of colistin combinations using a translational PK/PD approach. Further investigations of colistin combinations in animal infection models and patients are warranted to optimise colistin/doripenem combinations targeting isolates which are resistant to all antibiotics, including the last-line therapy colistin.

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Chapter Seven

Conclusions and future directions

7 Conclusions and future directions

Multidrug-resistance in Gram-negative bacteria is an emerging healthcare problem worldwide, resulting in an increasingly limited range of therapeutic options for treating infections caused by these pathogens (10). This alarming situation, coupled with the 'drying up' of the pharmaceutical development pipeline for anti-infective agents (10-11), necessitates optimising therapy with currently available agents. Colistin, a polymyxin antibiotic long relegated to the 'back shelf' due to early reports of a high incidence of nephro- and neuro-toxicity (54-56), retains activity against many of these MDR pathogens, often leaving it as the only therapeutic agent available (39, 45, 47, 524). Consequently use of colistin has increased dramatically over the last 5 years, especially in critically-ill patients (10, 39), and its place in therapy is being re-evaluated. Worryingly, resistance to colistin is beginning to emerge (45, 67, 69, 71-72). However, having never been subjected to the battery of drug development procedures now mandated by international drug regulatory authorities, there has been a lack of pharmacological information with which to inform rational use of colistin in order to maximise antibacterial activity and minimise toxicity and the development of resistance (39, 60). A greater understanding of the PK, PD and PK/PD relationships of colistin is essential in order to optimise use and prolong its therapeutic utility.

With this in mind, the studies described in this thesis were designed to investigate the PD and PK/PD relationships of colistin alone, and in combination, against *P. aeruginosa* in order to facilitate optimisation of its clinical use against this pathogen. The first study undertaken (Chapter 2) sought to determine, for the first time, the contribution to bacterial killing of both CMS (the sulphomethyl derivative of colistin and the form administered parenterally) and colistin. Using the reference strain *P. aeruginosa* ATCC 27853, the time-course of the killing effect achieved with CMS (and formed colistin) was very similar to that observed with colistin when added to achieve the same colistin concentration-time course resulting from the conversion of CMS. Killing with CMS did not begin until significant concentrations of colistin were

achieved, indicating that CMS and the partially sulphomethylated derivatives possess little, if any, antibacterial activity. As the time-course of antibacterial activity from CMS could be accounted for by the appearance of colistin, it was clearly demonstrated that antipseudomonal activity was due to formation of colistin, and that CMS is as an inactive prodrug of colistin. The likely explanation for this finding resides in differences in binding of CMS and colistin to cell surface LPS, the initial target of polymyxins, due to differences in net charge. This finding has important implications for standardisation of susceptibility studies (e.g., MIC measurement), as well as microbiological assays of 'colistin'. Given that the antibacterial activity of CMS results from its conversion to colistin, the PK/PD parameters used to describe activity of 'colistin' must be based on the concentrations of colistin present, not CMS. Consequently, all subsequent investigations in this thesis were conducted using colistin rather than CMS.

Confusion surrounding the optimal dosing regimen of CMS that maximises antibacterial activity and minimises the emergence of resistance formed the basis for investigations undertaken in Chapter 3. A one-compartment *in vitro* PK/PD model was employed to simulate the PK of colistin formation in humans with normal renal function administered three clinically relevant dosage regimens of CMS (8, 12, and 24 hourly). Studies were performed using a reference strain of *P. aeruginosa* (ATCC 27853) and a MDR mucoid clinical isolate (19056 muc); both strains were susceptible to colistin. All three regimens delivered approximately the same amount of colistin across a 24-h period (i.e., AUC/MIC was essentially the same for all regimens). The 8 hourly dosage regimen closely simulated the expected plasma unbound peak (fC_{\max}) and trough (fC_{\min}) concentrations of colistin at steady-state when CMS is administered 8 hourly according to the manufacturers recommendations (106-107). The investigations revealed overall bacterial killing and regrowth were generally similar across the three regimens. While this observation alone may suggest that the three regimens were equally effective, the emergence of resistant subpopulations increased as the dosage interval increased. For ATCC 27853, in the two regimens

which employed the greater dosage intervals (12 and 24 h), the emergence of resistance was substantially greater and occurred earlier than for the 8-hourly regimen. This result may be attributable to the very modest PAE of colistin against *P. aeruginosa* (63). As the dosage interval increased, colistin concentrations remained above the MIC for a smaller proportion of the treatment period. In the absence of a substantial PAE, the emergence of resistant subpopulations appears to be favoured by extended dosage intervals leading to protracted periods of colistin concentrations below the MIC. This sends a strong warning about the potential negative consequences of extended-interval dosing of colistin in patients (162, 387, 389).

As the study undertaken in Chapter 3 was not designed to elucidate the PK/PD index most closely related to antibacterial effect of colistin, a one-compartment *in vitro* PK/PD model was used to identify the PK/PD index (i.e., the area under the unbound concentration-time curve to MIC ratio [$fAUC/MIC$], the unbound maximal concentration to MIC ratio [fC_{max}/MIC], or the cumulative percentage of a 24-h period that unbound concentrations exceed the MIC [$fT_{>MIC}$]) that best predicts colistin efficacy against *P. aeruginosa* (Chapter 4). Such information is important for rational design of optimal dosing strategies. Dose fractionation was employed against three strains of *P. aeruginosa* (two reference strains, ATCC 27853 and PAO1, and a MDR mucoid clinical isolate, 19056 muc) with dosing regimens selected to maximally differentiate among the PK/PD indices under investigation. The $fAUC/MIC$ was most closely correlated with bacterial killing. Values for this index required to achieve various magnitudes of killing effect were subsequently determined. Respective values of $fAUC/MIC$ of ~25 and 35 for the reference strains were required to achieve 1- and 2-log reductions in the killing effect, which were in good agreement with animal studies (402). The corresponding values for the MDR clinical isolate were somewhat lower in the *in vitro* model than previously reported *in vivo*. Although the explanation for this difference is not known, it may relate to differences in growth dynamics between *in vitro*

and *in vivo* systems. It would greatly assist the rational design of colistin dosage regimens if the identified *fAUC/MIC* targets required to achieve various magnitudes of antibacterial effect were able to be compared with the *fAUC/MIC* values achieved in infected patients receiving currently recommended CMS dosage regimens. The present inability to undertake such a comparison results from a lack of information on unbound plasma colistin concentrations in infected patients. Future investigations into colistin plasma protein binding in infected patients will allow an assessment of the ability of current CMS dosage regimens to meet the identified targets, and assist in the design of optimised dosage regimens.

Two observations laid the foundation for studies undertaken in Chapters 5 and 6. First, eradication of colistin- or polymyxin B-susceptible Gram-negative bacteria with polymyxin monotherapy is difficult, often resulting in the selection of polymyxin-resistant subpopulations (74, 125-127, 385, 387, 394, Chapters 3 and 4). Secondly, PK studies in critically-ill patients and people with CF indicate that the currently recommended CMS dosage regimens are not able to generate plasma colistin concentrations that would be expected to be reliably efficacious (91-93, 95, 97). As nephrotoxicity is a dose-limiting adverse effect of colistin (occurring in up to ~50% of patients) (224, 235), increasing the daily dose of CMS in such patients may not be an option. As a consequence, studies were undertaken against *P. aeruginosa* to systematically investigate bacterial killing and emergence of resistance with colistin in combination with another antibiotic. These are the first studies to examine colistin combination therapy at multiple inocula ($\sim 10^6$ and $\sim 10^8$ cfu/mL), the latter to mimic the high bacterial densities found in some infections, and to monitor the emergence of resistance to colistin combination therapy. The study undertaken in Chapter 6 is the first to utilise an *in vitro* PK/PD model to investigate colistin in combination.

Colistin-heteroresistant *P. aeruginosa* was first identified in the study undertaken in Chapter 5. In both static (Chapter 5) and PK/PD (Chapter 6) time-kill investigations, colistin combined with

the carbapenem at clinically achievable concentrations increased bacterial killing against MDR (including colistin-resistant) and colistin-heteroresistant isolates at both inocula, even when ESBLs were present. Substantial differences were evident against the MDR colistin-resistant isolate, with bacterial eradication observed in the PK/PD model but not the static model, highlighting the importance of simulating PK profiles when examining PD responses. Differences in the emergence of colistin-resistant subpopulations were also evident between studies. Combination therapy in the static time-kill model against colistin-susceptible isolates generally had little effect on the proportion of colistin-resistant subpopulations when compared with colistin monotherapy, whereas resistant colonies generally emerged later and formed a substantially smaller proportion of the overall bacterial population with combination therapy in the PK/PD model. This difference may result from loss of the carbapenem due to degradation in the static experiments, and highlights once again the importance of PK/PD models in assessing activity and emergence of resistance of antimicrobial therapies.

Although the mechanism(s) underpinning the enhanced PD activity observed in Chapters 5 and 6 were not directly investigated as that was beyond the scope of those studies, based on the concepts of subpopulation synergy and mechanistic synergy as proposed previously (452) it may have been that one drug killed the resistant subpopulation(s) of the other drug, and *vice versa* (subpopulation synergy). Alternatively, or in addition to subpopulation synergy, both drugs may have acted on different cellular pathways to increase the rate or extent of killing of the other drug (mechanistic synergy); this latter mechanism more adequately explains the substantially enhanced activity of colistin/carbapenem combinations against the MDR colistin-resistant isolate given its near complete resistance to colistin. The elucidation of the mechanism(s) underpinning the enhanced PD activity observed with colistin/carbapenem combinations represents an opportunity for future investigations. Mechanism-based mathematical modeling will play an important role in predicting potential mechanisms and in proposing the most likely mechanisms.

Further investigations of colistin combinations in animal infection models and patients are also warranted to optimise colistin/carbapenem combinations targeting isolates resistant to all antibiotics, including colistin.

Further to the additional studies outlined in various paragraphs above, extending research in a number of other areas will be beneficial to support the work presented in this thesis. Firstly, investigations into the genetic mechanisms underpinning colistin resistance in *P. aeruginosa* are warranted, particularly the mechanism(s) by which colistin may induce resistance in an otherwise susceptible population. These investigations should include a consideration of the phenomenon of colistin heteroresistance and its underlying genetic basis. The use of genetically modified paired isolates, genetic complementation and, importantly, transcriptomics will play crucial roles in such investigations. Regarding transcriptomics, the use next-generation sequencing technology (RNA-Seq (552)) provides a new method for both mapping and quantifying transcriptomes. The application of RNA-Seq to investigations examining the genetic mechanisms involved in colistin resistance in *P. aeruginosa* and other bacterial species may provide valuable insights into this phenomenon. Additionally, as modifications to lipid A are known to be involved in colistin resistance in *P. aeruginosa*, application of lipidomics to the study of colistin resistance will be important. A greater understanding of these mechanisms will assist our understanding of how resistance to colistin develops and provide insight into dosing strategies to minimise resistance development. Secondly, studies on the concentrations of colistin achieved at infection sites other than plasma are urgently required. Finally, the development more generally of novel *in vitro* models or mechanism-based mathematical models capable of incorporating the effect of the immune system on bacterial killing would greatly improve our understanding of the synergy between the immune system and antibiotics (including colistin), providing greater insight into optimal dosing strategies in immunocompetent patients.

In summary, this thesis has shown CMS to be an inactive prodrug of colistin. The PD activity and emergence of colistin resistance to various CMS dosage regimens used clinically was reported. The first *in vitro* investigations specifically designed to elucidate the relationship between bacterial killing and PK/PD indices for colistin against any organism were undertaken, as well as the first systematic investigations into colistin combination therapy; the latter studies included the first examination of the emergence of colistin resistance with combination therapy. The studies reported here have increased understanding of the PD of colistin against *P. aeruginosa* and the findings are assisting in the design of optimal CMS dosing regimens that will maximise antimicrobial activity and minimise the emergence of colistin resistance.

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Appendix 1
Summary of clinical, animal and *in vitro* studies examining
colistin combination therapy

Table A1-1. Summary of clinical studies examining colistin monotherapy and combination therapy

Reference	Year/ Country	Study design	Type/site of infection	Pathogen	Therapeutic regimens including colistin dose (number of patients/ treatments in each treatment arm)	Monotherapy treatment cure/ improvement rates	Combination treatment cure/ improvement rates	Adverse effects	Comment
Conway <i>et al.</i> (160)	1997/ UK	Prospective	Respiratory exacerbations in patients with CF	<i>P. aeruginosa</i>	<p>CMS monotherapy: 160 mg CMS (2 million IU) 8-hourly IV (36 treatments).</p> <p>Combination therapy: 160 mg CMS (2 million IU) 8-hourly IV + one of AZT, AZL, PIP, CTZ, IMI or CIP administered according to the manufacturer's recommendations (35 treatments).</p>	36 (100%) of 36	35 (100%) of 35	Mild neurological symptoms: 37 events in 33 patients (severe in one patient) receiving CMS monotherapy; 37 events in 36 patients on dual therapy. Statistically significant decrease in CrCL in the combination group compared to baseline.	No significant differences between the groups except that more patients receiving dual therapy had a normal serum level of C-reactive protein. Differences in respiratory function tests and clinical scores between groups were not significant by day 12 of treatment.
Linden <i>et al.</i> (165)	2003/ USA	Prospective	Pneumonia, bacteremia, wound, intra- abdominal, endocarditis	MDR <i>P. aeruginosa</i>	<p>CMS monotherapy: ~2.7 – 13.3 mg/kg/day CMS (~33,000 – 167,000 IU/kg/day) IV (10 patients).</p> <p>Combination therapy: ~2.7 – 13.3 mg/kg/day CMS (~33,000 – 167,000 IU/kg/day) + AMI (4 patients) or antipseudomonal β-lactam (9 patients).</p>	6 (60%) of 10	8 (61.5%) of 13	No data supplied.	21 patients were critically-ill with ≥ 2 major organ system failures. There was no difference in favourable therapeutic outcome between CMS monotherapy and combination groups

Table A1-1. (Continued)

Falagas <i>et al.</i> (454)	2005/ Greece	Retrospective	Pneumonia, bacteremia, meningitis, UTI, CVC- related	<i>P. aeruginosa</i> <i>K. pneumoniae</i> (all isolates resistant to all anti- pseudomonal agents, including CMS)	CMS monotherapy: 80 mg CMS (1 million IU) 8-hourly IV (1 patient). Combination therapy: 80 mg CMS 12-hourly to 240 mg CMS 8- hourly (1 million IU 12- hourly to 3 million IU 8- hourly) IV + one of IMI, MER, OFX, GEN, AMP/SUL, TRI/SUL, CTZ or PIP/TAZ (5 patients).	0 (0%) of 1	4 (80%) of 5	No data supplied.	Microbiological as well as clinical cure achieved in 4 (80%) of 5 patients receiving combination therapy. The one patient receiving CMS monotherapy died due to meningitis.
Falagas <i>et al.</i> (221)	2006/ Greece	Retrospective	Pneumonia, UTI, abdominal, spondylodiscitis surgical site/skin and soft tissue, bacteremia, catheter- related, empirical	<i>P. aeruginosa</i> <i>A. baumannii</i> <i>K. pneumoniae</i> <i>S. maltophilia</i> <i>E. cloacae</i> <i>E. coli</i>	CMS monotherapy: mean (\pm SD) daily dose of CMS was 368 ± 184 mg (4.6 ± 2.3 million IU) (14 patients). Combination therapy: mean (\pm SD) daily dose of CMS was 440 ± 176 mg (5.5 ± 2.2 million IU) + MER (mean [\pm SD] daily dose was 4.8 ± 1.6 g) (57 patients).	12 (85.7%) of 14	39 (68.4%) of 57	4 (7.0%) of 57 patients receiving CMS + MER experienced nephrotoxicity; 3 of these patients died.	No difference in clinical response (cure and improvement) and occurrence of toxicity was found. In hospital mortality was less common among the CMS monotherapy group than among the CMS/MER group (0 [0%] of 14 vs. 21 [36.8%] of 57).
Tascini <i>et al.</i> (208)	2006/ Italy	Retrospective	Diabetic foot infection with/without osteomyelitis	MDR <i>P. aeruginosa</i>	CMS monotherapy: 80 mg CMS (1 million IU) 12-hourly IV or IM (4 patients). Combination therapy: 80 mg CMS (1 million IU) 12-hourly IV or IM + RIF (3 patients) or IMI (1 patient).	3 (75%) of 4	2 (50%) of 4	Renal insufficiency was observed in one patient after prolonged CMS monotherapy.	No difference in response or safety rates between mono- or combination therapy.

Table A1-1. (Continued)

Falagas <i>et al.</i> (67)	2008/ Greece	Retrospective	Bronchial secretions, bacteremia, CVC-related, surgical site, UTI, ascitic fluid, CNS	<i>P. aeruginosa</i> <i>K. pneumoniae</i> <i>A. baumannii</i> (all isolates resistant to all anti-pseudomonal agents, including CMS)	CMS monotherapy: CMS IV (4 patients). Combination therapy: CMS IV + one or more of MER, CIP, PIP/TAZ, TRI/SUL, GEN, CHL, RIF, AMP/SUL or AZT (18 patients). Doses of antibiotics were not specified.	2 (50%) of 4	9 (50%) of 18	No data supplied.	Infection-related mortality was 1 (25%) of 4 with CMS monotherapy and 5 (27.8%) of 18 with combination therapy.
Pintado <i>et al.</i> (187)	2008/ Spain	Prospective	Pneumonia, bacteremia, intra-abdominal, UTI, surgical site/soft tissue, catheter-related, meningitis, other	<i>P. aeruginosa</i> <i>K. pneumoniae</i> <i>A. baumannii</i> <i>E. aerogenes</i> <i>S. maltophilia</i> <i>E. cloacae</i> <i>E. coli</i> <i>A. anitratus</i>	CMS monotherapy: mean (\pm SD) daily dose of CMS was 3.76 ± 0.57 mg/kg ($\sim 47,000 \pm 7000$ IU/kg) IV (8 patients). Combination therapy: mean (\pm SD) daily dose of CMS was 4.52 ± 1.45 mg/kg ($\sim 56,000 \pm 18,000$ IU/kg) IV + either a β -lactam (8 patients), aminoglycoside (14), β -lactam and aminoglycoside (15), or CIP (8).	7 (87.5%) of 8	36 (69.2%) of 52	Despite the common use of combination therapy with aminoglycosides, nephrotoxicity during CMS therapy was observed in only 10.9% of patients, most of whom had pre-existing renal failure. No cases of clinically significant neurotoxicity were observed.	All MDR Gram-negative isolates were susceptible to CMS. A significant proportion of patients receiving combination therapy had severe infections; most patients receiving CMS monotherapy had non-severe infections such as UTI or surgical-site infections.

Table A1-1. (Continued)

Montero <i>et al.</i> (161)	2009/ Spain	Retrospective	Respiratory tract, bacteremia, UTI, skin and soft tissue, otitis, arthritis	MDR <i>P. aeruginosa</i>	Total daily dose of CMS (median [range]): <u>IV administration</u> 240 mg CMS [120 – 480 mg] (3 million IU [1.5 – 6]) <u>Nebulised administration</u> 120 mg CMS [80 – 480 mg] (1.5 million IU [1 – 6 million IU]). CMS doses (or route of administration) for mono- and combination groups not specified. Combination therapy: CMS + one or more of an aminoglycoside, β -lactam, quinolone, or carbapenem; doses not specified.	27 (73%) of 37	41 (71.9%) of 57 with an aminoglycoside; 18 (72.0%) of 25 with a β -lactam; 6 (75.0%) of 8 with a quinolone; 5 (62.5%) of 8 with a carbapenem.	No cases of neurotoxicity were detected, but 10 patients (8.3%) developed nephrotoxicity.	Across mono- and combination therapy groups those patients receiving a relatively higher dose of CMS (>240 mg/day [>3 million IU/day]) had a favourable clinical response in 17 (85%) of 20 cases; in comparison, 69.3% of patients whose CMS dose was \leq 240 mg/day (\leq 3 million IU/day) had a favourable clinical response.
Tsioutis <i>et al.</i> (455)	2010/ Greece	Prospective	Respiratory tract, bacteremia, UTI, surgical site/skin and soft tissue	<i>P. aeruginosa</i> <i>K. pneumoniae</i> <i>A. baumannii</i>	CMS monotherapy: CMS IV (1 patient). Combination therapy: CMS IV + another agent (either CIP, DOX, MER or RIF; 9 patients). Doses of antibiotics were not specified.	1 (100%) of 1	6 (66.7%) of 9	No data supplied.	3 (33.3%) of 9 receiving CMS combination therapy died.

Table A1-1. (Continued)

Falagas <i>et al.</i> (453)	2010/ Greece	Retrospective	Pneumonia, UTI, bacteremia, surgical site/soft tissue, catheter- related, CSF, other	<i>P. aeruginosa</i> <i>K. pneumoniae</i> <i>A. baumannii</i> others	Mean daily doses of CMS (IV) not stated, although the daily dose range was at least 240 – 720 mg CMS (3 – 9 million IU). Other antimicrobials for combination therapy (doses not stated) included MER, IMI, PIP/TAZ, AMP/SUL, aminoglycosides, cephalosporins, AZT, and CIP. 36 patients received CMS monotherapy and 222 patients combination therapy.	30 (83.3%) of 36	CMS + MER: 135 (83.3%) of 162; CMS + PIP/TAZ: 11 (64.7%) of 17; CMS + AMP/SUL: 9 (75.0%) of 12; CMS + other agents: 19 (61.3%) of 31	Nephrotoxicity in 10 (19.2%) of 52	The proportion of polymyxin-only pathogens was 135 (52.3%) of 258; the remainder were susceptible to CMS and at least one other antimicrobial agent. In patients with infections caused by polymyxin-only- susceptible pathogens, 18 (90%) of 20 patients treated with CMS monotherapy were cured compared with 70 (83.3%) of 84 patients treated with CMS + MER and 17 (54.8%) of 31 patients treated with CMS + other antimicrobial agents. The average daily dose of CMS was an independent factor for mortality; there was 64 mg CMS (0.8 million IU) difference in the average CMS dose between survivors and deceased patients (survivors received a higher dose).
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AMI, amikacin; AMP/SUL, ampicillin/sulbactam; AZL, azlocillin; AZT, aztreonam; CHL, chloramphenicol; CIP, ciprofloxacin; CMS, colistin methanesulphonate (sodium); CTZ, ceftazidime; DOX, doxycycline; GEN, gentamicin; IMI, imipenem; MER, meropenem; OFX, ofloxacin; PIP, piperacillin; PIP/TAZ, piperacillin/tazobactam; RIF, rifampicin; TRI/SUL, trimethoprim/sulfamethoxazole; CF, cystic fibrosis; CNS, central nervous system; CSF, cerebrospinal fluid; CrCL, creatinine clearance; CVC, central venous catheter; IM, intramuscular; IV, intravenous; IU, international units; MDR, multidrug-resistant; UTI, urinary tract infection; VAP, ventilator-associated pneumonia.

Table A1-2. Summary of animal studies comparing colistin monotherapy and combination therapy

Reference	Year/ Country	Study design, animals and type of infection	Pathogen (number of isolates)	Antimicrobials	Therapeutic regimens (number of animals in each group)	Monotherapy treatment outcome	Combination treatment outcome	Comment
Giacometti <i>et al.</i> (461)	2003/ Italy	Septic shock rat model	<i>E. coli</i> (1)	'COL' [*] , PIP	Controls (15); COL 1 mg/kg (15); PIP 60 mg/kg (15); COL + PIP at monotherapy doses (15). Single doses of antibiotics were administered.	Mortality at 48 h: Controls, 14 (93.3%) of 15; COL, 5 (33.3%) of 15; PIP, 5 (33.3%) of 15	Mortality at 48 h: 0 (0%) of 15	Rats received an IP inoculum containing 2×10^6 cfu. The combination substantially reduced bacterial counts obtained from blood samples in surviving animals. COL or COL + PIP significantly reduced endotoxemia in comparison with PIP.
Montero <i>et al.</i> (457)	2004/ Spain	Mouse pneumonia model	MDR <i>A. baumannii</i> (1; contained an OXA-24-like carbapenemase conferring high level IMI resistance)	CMS, RIF	Controls (15); CMS 10 mg/kg 6-hourly IP (8); RIF 25 mg/kg/day (single dose) IP (8); CMS + RIF at monotherapy dosage regimens (8). Treatments were continued for 48 h.	Survival at 48 h: Controls, 7 (46.7%) of 15; CMS, 7 (87.5%) of 8; RIF, 8 (100%) of 8	Survival at 48 h: 8 (100%) of 8	50 μ L of an inoculum of 10^8 cfu/mL was administered by intratracheal installation. The combination did not provide any significant difference in lung bacterial counts and bacteremia (0%) in comparison with RIF monotherapy.

* Colistin (sulphate) or CMS (sodium) not specified

Table A1-2. (Continued)

Cirioni <i>et al.</i> (460)	2007/ Italy	Mouse model of sepsis	<i>P. aeruginosa</i> (2) ATCC 27853 1 MDR clinical isolate	'COL' [*] , IMI	Controls (20); COL 1 mg/kg IV (20); IMI 20 mg/kg IV (20); COL + IMI at monotherapy doses (20). Single doses of antibiotics were administered.	Mortality at 72 h: <u>ATCC 27853</u> Controls, 20 (100%) of 20; COL, 8 (40%) of 20; IMI, 6 (30%) of 20 <u>MDR clinical isolate</u> Controls, 20 (100%) of 20; COL, 6 (30%) of 20; IMI, 16 (80%) of 20	Mortality at 72 h: <u>ATCC 27853</u> 2 (10%) of 20 <u>MDR clinical isolate</u> 3 (15%) of 20	IV inoculum of 2×10^7 cfu. The combination produced significantly lower mortality and bacteraemia than monotherapy. COL (alone or in combination) resulted in marked decreases ($p < 0.05$) of LPS, TNF- α and IL-6 plasma concentrations compared to controls.
Cirioni <i>et al.</i> (459)	2007/ Italy	Rat model of sepsis	<i>P. aeruginosa</i> (2) ATCC 27853 1 MDR clinical isolate	'COL' [*] , RIF	Controls (15); COL 1 mg/kg (15); RIF 10 mg/kg (15); COL + RIF at monotherapy doses (15). Drug regimens administered either immediately or 6 h after bacterial challenge. Single doses of antibiotics were administered.	Mortality at 72 h: <u>ATCC 27853</u> With immediate treatment: Controls, 15 (100%) 15; COL, 4 (26.7%) of 15; RIF, 14 (93.3%) of 15. With 6 h delayed treatment: Controls, 15 (100%) of 15; COL, 3 (20%) of 15; RIF 15 (100%) of 15 <u>MDR clinical isolate</u> With immediate treatment: Controls, 15 (100%) of 15; COL, 8 (53.3%) of 15; RIF, 15 (100%) of 15. With 6 h delayed treatment: Controls, 15 (100%) of 15; COL, 9 (60%) of 15; RIF, 15 (100%) of 15	Mortality at 72 h: <u>ATCC 27853</u> 1 (6.7%) of 15 with immediate treatment; 1 (6.7%) of 15 with 6 h delayed treatment <u>MDR clinical isolate</u> 4 (26.7%) of 15 with immediate treatment; 4 (26.7%) of 15 with 6 h delayed treatment	Rats received an IP inoculum containing 2×10^{10} cfu. COL + RIF resulted in a significant reduction in bacterial counts compared to COL monotherapy (the most active agent) despite no significant difference in positive blood cultures and mortality between the two groups. COL alone or in combination reduced plasma LPS and TNF- α levels, whereas no differences were observed between RIF- treated and untreated groups.

* Colistin (sulphate) or CMS (sodium) not specified

Table A1-2. (Continued)

Pantopoulou <i>et al.</i> (462)	2007/ Greece	Neutropenic rat thigh infection model	MDR <i>A. baumannii</i> (1)	'COL' [*] , RIF	Controls (20); COL 3 mg/kg IM (20); RIF 5 mg/kg IV (20); COL + RIF at monotherapy doses (20). Single doses of antibiotics were administered.	Median survival: Controls, 2 days; COL, 4 days; RIF, 2.5 days Mortality after 6 days: Controls, 10 (100%) of 10; COL, 10 (100%) of 10; RIF, 10 (100%) of 10	Median survival: 4 days Mortality after 6 days: 7 (70%) of 10	IM cfu inoculum not specified. All animals died after 11 days following bacterial challenge. Statistically significant differences in survival were only found between control and COL, and control and COL + RIF groups. Statistically significant decreases in numbers of bacteria were found in blood and liver of the COL + RIF versus control groups.
Aoki <i>et al.</i> (410)	2009/ Japan	Mouse pneumonia model	<i>P. aeruginosa</i> (1)	CMS, IMI, RIF	Each group contained 14 – 16 mice; the precise number in each group was not specified. Controls, CMS 20 mg/kg/day SC or 10 mg/kg/day INS (both 12-hourly); IMI 60 mg/kg/day 12-hourly; RIF 25 mg/kg/day 24- hourly; CMS + IMI or CMS + RIF at monotherapy doses. Treatments were continued for 48 h.	All control, CMS- (SC or INS), RIF- and IMI- monotherapy-treated mice died within 42 h following infection.	At an inoculum of ~2 – 2.5 × 10 ⁶ cfu/mouse, CMS (INS) + RIF or IMI increased survival to 75% and 62.5% at 72 h, respectively. A clear discrepancy in survival was observed between mice treated with CMS (INS) + RIF and CMS (SC) + RIF (100% vs. 14%). CMS (INS) + IMI was also superior to CMS (SC) + IMI. Similar results were observed at the lower inoculum.	Two inocula were used: 2 – 2.5 × 10 ⁶ and 1 – 1.5 × 10 ⁶ cfu/mouse. INS CMS produced significant reductions in lung inflammatory cytokines.

* Colistin (sulphate) or CMS (sodium) not specified

Table A1-2. (Continued)

Pachón-Ibáñez <i>et al.</i> (458)	2010/ Spain	Mouse pneumonia model and rabbit meningitis model	MDR <i>A. baumannii</i> (1)	CMS, RIF	<p><u>Pneumonia model:</u> Control (15); CMS 60 mg/kg/day 8-hourly IM (15); RIF 100 mg/kg/day 6-hourly IP (14); CMS + RIF at monotherapy doses (16). Treatments were continued for 72 h.</p> <p><u>Meningitis model:</u> Control (8); CMS 12 mg/kg IM (8); RIF 25 mg/kg IV (8); CMS + RIF at monotherapy doses (8). Single doses of antibiotics were administered.</p>	<p><u>Pneumonia model:</u> Survival at 72 h: Controls, 0 (0%) of 15; CMS, 6 (40%) of 15; RIF, 10 (71.4%) of 14</p> <p><u>Meningitis model:</u> Median bacterial CSF concentrations (log₁₀ cfu/mL) at 6 h: RIF, 3.5; CMS, 4.3</p>	<p><u>Pneumonia model:</u> Survival at 72 h: 7 (43.8 %) of 16</p> <p><u>Meningitis model:</u> Median bacterial CSF concentration (log₁₀ cfu/mL) at 6 h: 1.3.</p>	<p>In the pneumonia model 50 µL of an inoculum of 10⁸ cfu/mL was administered intratracheally. Meningitis was induced by inoculating 200 µL of a suspension containing ~10⁷ cfu/mL into the CSF. Bacterial clearance from the lungs in the pneumonia model was substantially reduced with both CMS and RIF monotherapy. CMS + RIF did not increase bacterial clearance from the lungs over RIF alone. CMS + RIF improved bacterial clearance from the blood over that of monotherapy.</p>
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CMS, colistin methanesulphonate (sodium); COL, colistin (sulphate); IMI, imipenem; PIP, piperacillin; RIF, rifampicin; cfu, colony forming units; CSF, cerebral spinal fluid; IM, intramuscular; INS, intranasal; IP, intraperitoneal; IL-6, interleukin-6; IV, intravenous; LPS, lipopolysaccharide; MDR, multidrug-resistant; SC, subcutaneous; TNF- α , tumour necrosis factor- α .

Table A1-3. Summary of *in vitro* studies using the time-kill method comparing colistin/CMS (or polymyxin B) monotherapy and combination therapy

Reference	Year/ Country	Pathogen (number of isolates)	Antimicrobials	Colistin monotherapy vs. colistin combination therapy	Comment
Giamarellos-Bourboulis <i>et al.</i> (553)	2001/ Greece	MDR <i>A. baumannii</i> (39)	CMS alone RIF alone CMS + RIF	All isolates susceptible to CMS (MIC ≤0.03 – 2 mg/L), although it is unclear whether MIC determinations were performed with CMS or COL. Synergy with CMS (1× MIC) + RIF (2 mg/L) was found in 6 (15.4%) and 20 (51.3%) of 39 isolates at 6 and 24 h, respectively; the corresponding results for CMS (4× MIC) + RIF (2 mg/L) were 6 (15.4%) and 26 (66.7%) of 39 isolates. No synergy was found at 2 and 4 h.	Initial inoculum: 5 × 10 ⁵ cfu/mL The MIC ₅₀ and MIC ₉₀ of 'COL' were reported as 0.12 and 1 mg/L, respectively; however, it is unclear whether MIC determinations were performed with CMS or COL. CMS + RIF prevented regrowth at 6 h; regrowth was observed at 6 h with CMS monotherapy.
Giamarellos-Bourboulis <i>et al.</i> (554)	2002/ Greece	<i>S. maltophilia</i> (24)	CMS alone RIF alone TRI/SUL alone CMS + RIF CMS + TRI/SUL	All isolates resistant to RIF and TRI/SUL; susceptibilities to CMS/COL not stated. Synergy with CMS (1× MIC) + RIF (2 mg/L) was found in 16 (66.7%), 14 (58.3%), 14 (58.3%) and 13 (54.2%) of 24 isolates at 2, 4, 6, and 24 h, respectively; the corresponding values with CMS (4× MIC) + RIF (2 mg/L) were 14 (58.3%), 14 (58.3%), 14 (58.3%) and 15 (62.5%), respectively. Synergy with CMS (1× MIC) + TRI/SUL (2/38 mg/L) was found in 2(8.3%), 1 (4.2%), 3 (12.5%) and 4 (16.7%) of 24 isolates at 2, 4, 6, and 24 h, respectively; the corresponding values with CMS (4× MIC) + TRI/SUL (2/38 mg/L) were 5 (20.8%), 6 (25.0%), 6 (25.0%) and 10 (41.7%), respectively.	Initial inoculum: 1 × 10 ⁶ cfu/mL The MIC ₅₀ and MIC ₉₀ of colistin were reported as 4 and 16 mg/L, respectively; however, it is unclear whether MIC determinations were performed with CMS or COL. Regrowth observed at 24 h with CMS monotherapy was prevented with both combinations.
Giamarellos-Bourboulis <i>et al.</i> (555)	2003/ Greece	MDR <i>P. aeruginosa</i> (17)	CMS alone RIF alone CMS + RIF	'COL' MICs 1 – 128 mg/L, although it is unclear whether MIC determinations were performed with CMS or COL. Synergy between CMS (2 mg/L) and RIF (2 mg/L) was found in 4 (23.5%), 6 (35.3%), 7 (41.7%) and 2 (11.8%) of 17 isolates after 2, 4, 6 and 24 h, respectively. Synergy was found in 3 (33.3%) of 9 isolates with a 'COL' MIC ≥8 mg/L; a 'COL' MIC ≥8 was considered susceptible.	Initial inoculum: 1 × 10 ⁶ cfu/mL The level of the MIC of 'COL' did not correlate with the possibility of occurrence of synergy with RIF.

Table A1-3. (Continued)

Gunderson <i>et al.</i> (387) [†]	2003/ USA	MDR <i>P. aeruginosa</i> (2)	COL alone CTZ alone COL + CTZ COL + CIP	The MIC of COL for each isolate was 0.125 mg/L; both isolates were resistant to CTZ and CIP. COL (C_{max} 6 or 18 mg/L, 24-hourly administration; $t_{1/2}$ 3 h) + CTZ (50 mg/L continuous infusion) was reported as synergistic at 24 h. The combination with COL at a C_{max} of 18 mg/L was only slightly more effective than with the combination containing COL at a C_{max} of 6 mg/L. COL (C_{max} 6 or 18 mg/L, 24-hourly administration; $t_{1/2}$ 3 h) + CIP (C_{max} 5 mg/L, 12-hourly administration; $t_{1/2}$ 3 h) generally produced poorer bacterial killing.	Initial inoculum: 1×10^6 cfu/mL Although synergy with COL + CTZ was reported, only changes in \log_{10} cfu/mL between COL monotherapy and combination therapy were considered; when data for CTZ monotherapy (which was performed on only one of the two isolates) is considered, synergy was not observed.
Tascini <i>et al.</i> (480)	2004/ Italy	MDR <i>P. aeruginosa</i> (2)	'COL' [*] alone RIF alone 'COL' + RIF	COL MICs of 4 mg/L for each isolate, although it is unclear whether MIC determinations were performed with CMS or COL. COL (0.5× MIC) and RIF (1/8× MIC) as monotherapy produced little antimicrobial activity with regrowth close to control values by 6 h. The combination at the same concentrations was bactericidal and the effect was prolonged for 12 h.	Initial inoculum: 1×10^6 cfu/mL
Yoon <i>et al.</i> (556)	2004/ USA	MDR <i>A. baumannii</i> (8)	PB + IMI PB + RIF PB + IMI + RIF	Seven of 8 isolates were susceptible to PB (MICs 1 – 8 mg/L); all isolates were resistant to IMI. Experiments were conducted using concentrations of 0.25 mg/L PB, 8 mg/L IMI, and 0.5 mg/L of RIF. Results of monotherapy were not reported. No viable bacteria were detected at 24 h with the triple combination (PB + IMI + RIF); at 24 h, one isolate showed regrowth with PB + IMI, and one isolate showed regrowth with PB + RIF.	Initial inoculum not specified. Although the combinations of PB + IMI + RIF, PB + IMI and PB + RIF were reported as bactericidal in 8, 7 and 7 of 8 isolates, respectively, it is not reported whether these combinations were synergistic or demonstrated improved activity over monotherapy.
Cirioni <i>et al.</i> (460)	2007/ Italy	<i>P. aeruginosa</i> (2)	'COL' [*] alone IMI alone 'COL' + IMI	COL and IMI MICs of 4 and 0.5 mg/L for the reference strain, and 8 and 32 mg/L for a MDR isolate, respectively; it is unclear whether MIC determinations were performed with CMS or COL. Synergy was reported between COL and IMI for both strains at concentrations of 0.25× and 0.5× MIC for each drug.	Initial inoculum: 5×10^5 cfu/mL Log changes or graphical data were not provided.

[†] Utilised a one-compartment PK/PD model

^{*} Colistin (sulphate) or CMS (sodium) not specified

Table A1-3. (Continued)

Song <i>et al.</i> (557)	2007/ Korea	IMI-resistant <i>A. baumannii</i> (8)	CMS alone CMS + RIF	Based on MICs 100% and 95% of isolates were considered susceptible to CMS and RIF. CMS was bactericidal against all isolates at 4 and 8× MIC, usually beginning around 8 h following inoculation. RIF monotherapy was not performed. CMS (1× MIC) + RIF (1× MIC) was synergistic and bactericidal against all isolates, with a mean decrease of $4.29 \pm 1.47 \log_{10}$ cfu/mL after 8 h.	Initial inoculum: 1×10^5 cfu/mL MICs were determined to 'COL' using CMS, which is inappropriate (see Chapter 2).
Tan <i>et al.</i> (488)	2007/ Singapore	IMI-resistant <i>A. baumannii</i> (13)	COL alone MIN alone COL + MIN	All isolates were susceptible to COL (MICs 0.5 – 2 mg/L); 70% of isolates were susceptible to MIN. For isolates susceptible to both antibiotics, a final concentration of 1× MIC was used for each antibiotic. MIN-resistant strains were tested at a MIN concentration of 4 mg/L. Monotherapy with COL or MIN failed to demonstrate bactericidal activity. COL + MIN was rapidly bactericidal. Synergy was detected in 12 (92.3%) of 13 isolates at 24 h (and in 3 [75%] of 4 MIN-resistant isolates at 24 h).	Initial inoculum: 5×10^5 cfu/mL With monotherapy regrowth occurred at 24 h with both COL and MIN. For COL + MIN there was minimal evidence of bacterial regrowth at 24 h. Results of concurrent Etest synergy studies showed no agreement with those of the time-kill method.
Tripodi <i>et al.</i> (478)	2007/ Italy	MDR <i>A. baumannii</i> (9)	'COL' * alone IMI alone RIF alone AMP/SUL alone 'COL' + IMI 'COL' + IMI + RIF 'COL' + AMP/SUL 'COL' + AMP/SUL +RIF	All isolates were susceptible to COL (MIC 2 mg/L) and resistant to IMI and AMP/SUL; susceptibility to RIF was variable. Concentrations employed were: COL, 6 mg/L; IMI, 20 mg/L; AMP/SUL, 50 mg/L; and RIF, 5 mg/L. As monotherapy only COL showed a bactericidal effect. In general, the activity of each antibiotic combination showed indifference, with an activity of that similar to COL monotherapy.	Initial inoculum: 1×10^5 cfu/mL It is unclear whether 'COL' MICs were performed with COL or CMS. The high concentration of COL employed (6 mg/L) may not have allowed synergy to be observed.

* Colistin (sulphate) or CMS (sodium) not specified

Table A1-3. (Continued)

Pankuch <i>et al.</i> (476)	2008/ USA	<i>P. aeruginosa</i> (51) <i>A. baumannii</i> (52)	'COL' * alone MER alone 'COL' + MER	For <i>P. aeruginosa</i> , COL and MER MIC ranges were 0.25 – 4 mg/L and 0.12 – 256 mg/L, respectively; the equivalent values for <i>A. baumannii</i> were 0.25 – 128 mg/L and 0.12 – 256 mg/L. <i>P. aeruginosa</i> : Synergy achieved against 26 (51%) of 51 isolates at 3 h with sub-MIC COL and MER concentrations of 0.12 – 1 and 0.03 – 64 mg/L, respectively. At 6 h synergy achieved against 23 (45.1%) of 51 isolates with sub-MICs of COL (0.12 – 1 mg/L) and MER (0.06 – 64 mg/L). After 12 h synergy achieved against 24 (47.1%) of 51 isolates with sub-MICs of COL (0.12 – 1 mg/L) and MER (0.03 – 4 mg/L). At 24 h synergy achieved against 13 (25.5%) of 51 isolates with sub-MICs of COL (0.12 – 1 mg/L) and MER (0.06 to 8 mg/L). <i>A. baumannii</i> : Synergy achieved against 20 (38.5%) of 52 isolates at 3 h with sub-MIC COL and MER concentrations of 0.03 – 16 and 0.03 – 64 mg/L, respectively. At 6 h synergy achieved against 39 (75%) of 52 isolates with sub-MICs of COL (0.06 – 8 mg/L) and MER (0.03 – 64 mg/L). After 12 h synergy achieved against 50 (96.2%) of 52 isolates with sub-MICs of COL (0.06 – 16 mg/L) and MER (0.03 – 64 mg/L). At 24 h synergy achieved against 49 (94.2%) of 52 isolates with sub-MICs of COL (0.062 – 8 mg/L) and MER (0.03 to 64 mg/L).	Initial inoculum: >5 × 10 ⁵ cfu/mL It is unclear whether 'COL' MICs were performed with COL or CMS.
Aoki <i>et al.</i> (410)	2009/ Japan	MDR <i>P. aeruginosa</i> (1)	'COL' * alone 'COL' + AMI, CIP, IMI or RIF	COL MIC 2 mg/L, although it is unclear whether MIC determinations were performed with CMS or COL. It is also unclear whether monotherapy with AMI, CIP, IMI or RIF was performed. Experiments were conducted using 1× MIC of COL plus 0.5× MIC of other drugs, 1× MIC of COL plus 0.25× MIC of other drugs, and 0.5× MIC of COL plus 0.5× MIC of other drugs. COL + RIF or IMI was synergistic; COL + CIP or AMI was indifferent.	Initial inoculum: 5 × 10 ⁵ cfu/mL No definition of synergy was provided. Given it is unclear whether monotherapy was performed with drugs other than COL it is difficult to determine what the reported 'synergy' means. LPS release was also examined. Sub-MICs of COL significantly reduced release of LPS in a concentration-dependent manner. The amounts of LPS released were <10% of the control when grown in the presence of 1 mg/L COL, despite no change in viable bacteria.

* Colistin (sulphate) or CMS (sodium) not specified

Table A1-3. (Continued)

Pankey <i>et al.</i> (558)	2009/ USA	MDR <i>A. baumannii</i> (8)	PB alone MER alone PB + MER	All isolates were susceptible to PB (Etest MICs 0.5 mg/L) and resistant to MER (Etest MICs 24 – >32 mg/L). PB was used at 0.25, 0.5 and 1× MIC. MER was used at 1× MIC; when the MER Etest MIC was >32 mg/L, an MER concentration of 32 mg/L was used. PB and MER monotherapy showed minimal antibacterial activity. Synergy was demonstrated at 24 h against all 8 isolates with all combinations of PB (0.25, 0.5 and 1× MIC) + MER (1× MIC).	Initial inoculum: 1×10^5 cfu/mL
Souli <i>et al.</i> (475)	2009/ Greece	MBL-producing <i>K. pneumoniae</i> (42)	COL alone IMI alone COL + IMI	All isolates carried a <i>bla</i> _{VIM-1} -type gene. COL and IMI MICs ranged from 0.19 – 256 mg/L and 0.75 – >32 mg/L, respectively. COL (5 mg/L) + IMI (10 mg/L) was synergistic against 3 (37.5%) of 8 isolates susceptible to both COL and IMI, 9 (56.3%) of 16 IMI-resistant, COL-susceptible isolates, and 2 (13.3%) of 15 IMI- and COL-resistant isolates. The combination was antagonistic against 7 (46.7%) of 15 and 3 (100%) of 3 IMI- and COL-resistant, and IMI-susceptible and COL-resistant isolates, respectively. Against non-COL-susceptible isolates synergy was observed only against isolates with COL MICs of 3 – 4 mg/L.	Initial inoculum not specified. For 7 of 12 isolates initially susceptible to COL, a COL-resistant clone (MICs 64 – >256 mg/L) was selected after incubation with the tested combination. Conversely, no resistance to IMI was detected in 4 isolates initially susceptible to IMI that showed regrowth after 24 h.

Table A1-3. (Continued)

Pankuch <i>et al.</i> (481)	2010/ USA	<i>P. aeruginosa</i> (25) <i>A. baumannii</i> (25)	'COL' * alone DOR alone 'COL' + DOR	For <i>P. aeruginosa</i> , COL and DOR MICs ranged from 0.12 – 32 mg/L and 0.12 – 512 mg/L, respectively; the equivalent values for <i>A. baumannii</i> were 0.5 – 32 mg/L and 0.25 – 128 mg/L. <u><i>P. aeruginosa</i></u> : Synergy against 11 (44%) of 25 isolates at 3 h with COL and DOR concentrations of 0.12 – 1.0 mg/L and 0.03 – 128 mg/L, respectively; at 6h, 17 (68%) of 25 isolates showed synergy with COL (0.12 – 8.0 mg/L) and DOR (0.03 – 128 mg/L); at 12 h, 18 (72%) of 25 isolates showed synergy with COL (0.12 – 16 mg/L) and DOR (0.03 – 128 mg/L); after 24 h, 19 (76%) of 25 isolates showed synergy with COL (0.12 – 16 mg/L) and DOR (0.03 – 128 mg/L). <u><i>A. baumannii</i></u> : Synergy against 2 (8%) of 25 isolates at 3 h with COL (0.25 – 2 mg/L) and DOR (0.12 – 8.0 mg/L); at 6h, 8 (32%) of 25 isolates showed synergy with COL (0.12 – 4 mg/L) and DOR (0.12 – mg/L); at 12 h, 23 (92%) of 25 isolates showed synergy with COL (0.12 – 8.0 mg/L) and DOR (0.06 – 32 mg/L); after 24 h, 25 (100%) of 25 isolates showed synergy with COL (0.12 – 16 mg/L) and DOR (0.06 – 32 mg/L).	Initial inoculum: 1×10^6 cfu/mL
Sun <i>et al.</i> (484)	2010/ USA	MDR <i>P. aeruginosa</i> (1; DOR-resistant)	COL alone DOR alone TOB alone COL + DOR + TOB	For monotherapy, concentrations used were $1 \times$ MIC for each antibiotic. The most active single agent was TOB, although it did not appreciably reduce bacterial growth below the starting inoculum at any time across 24 h. The triple combination (COL + DOR + TOB) at concentrations of $0.5 \times$ MIC was similar to TOB monotherapy at $1 \times$ MIC. The triple combination at concentrations of $1 \times$ MIC was rapidly bactericidal and synergistic at 8 h; at 24 h, the growth difference between TOB monotherapy and triple therapy was $7.3 \log_{10}$ cfu/mL.	Initial inoculum: 1×10^6 cfu/mL

* Colistin (sulphate) or CMS (sodium) not specified

Table A1-3. (Continued)

Urban <i>et al.</i> (483)	2010/ USA	<i>P. aeruginosa</i> (5) <i>A. baumannii</i> (5) <i>K. pneumoniae</i> (5) <i>E. coli</i> (5)	PB + DOR PB + RIF PB + DOR + RIF	All isolates were resistant to DOR; the majority of isolates were MDR. Monotherapy with PB, DOR, or RIF at 0.25× MIC was not bactericidal. PB + DOR + RIF at 0.25× MIC for each antibiotic was bactericidal for 5 (100%) of 5 <i>P. aeruginosa</i> , 3 (60%) of 5 <i>A. baumannii</i> , 4 (80%) of 5 <i>K. pneumoniae</i> and 5 (100%) of 5 <i>E. coli</i> isolates. PB + DOR at 0.25× MIC for each antibiotic was bactericidal for 1 (20%) of 5 <i>P. aeruginosa</i> , 1 (20%) of 5 <i>A. baumannii</i> , 1 (20%) of 5 <i>K. pneumoniae</i> and 4 (80%) of 5 <i>E. coli</i> isolates. PB + RIF at 0.25× MIC for each antibiotic was bactericidal for 1 (20%) of 5 <i>P. aeruginosa</i> , 2 (40%) of 5 <i>A. baumannii</i> , 1 (20%) of 5 <i>K. pneumoniae</i> and 2 (40%) of 5 <i>E. coli</i> isolates.	Initial inoculum not specified. Bactericidal activity was achieved at 24 h in 85% of all bacteria using combinations of PB + DOR + RIF, in 30% of all bacteria with PB + DOR and in 25% of all bacteria with PB + RIF at 0.25× MIC.
Gordon <i>et al.</i> (485)	2010/ UK	MDR <i>A. baumannii</i> (5)	'COL' * alone VAN alone 'COL' + VAN	All isolates reported as susceptible to COL. Concentrations used were COL 1 mg/L and VAN 20 mg/L. Monotherapy with COL was initially bactericidal against all isolates, with rapid regrowth after 4 h. VAN was virtually inactive as monotherapy. COL + VAN resulted in rapid bactericidal activity, with regrowth occurring after 48 h in 1 (20%) of 5 isolates.	Initial inoculum: $1 \times 10^5 - 10^6$ cfu/mL Electron microscopy showed significant differences between cells exposed to colistin and unexposed cells. The surface of colistin-exposed cells exhibited increased topographic variability and appeared rougher, with visible pits. There was also a greater degree of debris surrounding many of the colistin-exposed cells.
Liang <i>et al.</i> (479)	2011/ China	MDR <i>A. baumannii</i> (4)	COL alone MER alone MIN alone RIF alone COL + MER COL + MIN COL + RIF	COL MICs 0.25 – 0.5 mg/L. MICs for MER, MIN and RIF were 16 – 128 mg/L, 2 – 8 mg/L, and 4 – 16 mg/L, respectively. As monotherapy COL (0.5× MIC), MER (8 mg/L), MIN (1 mg/L) or RIF (0.06 mg/L) did not show bactericidal activity. Complete killing of all isolates was achieved within 2 – 6 h when high concentrations of MER (16 mg/L), MIN (2 mg/L) or RIF (0.25 mg/L) were used in combination with COL (0.5× MIC). When combined with COL at 0.5× MIC, the lowest concentrations of MER, MIN and RIF to achieve a synergistic effect were determined: for MER, 4 mg/L was sufficient for synergy (3 isolates); for MIN, 1 mg/L showed synergy against all 4 isolates; the lowest concentration of RIF tested (0.06 mg/L) also showed synergy (number of isolates not specified).	Initial inoculum: 1×10^6 cfu/mL

* Colistin (sulphate) or CMS (sodium) not specified

Table A1-3. (Continued)

Lin <i>et al.</i> (388)	2010/ Taiwan	<i>E. cloacae</i> (1)	COL alone IMI alone COL + IMI	This isolate was COL- and IMI-susceptible (MICs 1.0 and 0.5 mg/L, respectively). COL monotherapy at 2× and 4× MIC (2 and 4 mg/L) and IMI monotherapy at 2× MIC (1 mg/L) resulted in regrowth to control levels by 24 – 48 h at both inocula; IMI monotherapy at 4× MIC (2 mg/L) resulted in rapid killing and suppression of regrowth for 48 h. COL + IMI (both at 2× MIC) resulted in rapid killing at both inocula. No regrowth was observed by 48 h at the low inoculum (the combination being synergistic), but regrowth commenced at ~8 h at the high inoculum and approached that of the growth control by ~48 h; synergy was not evident at the high inoculum.	Two initial inocula were used: 5×10^5 and 6.24×10^6 cfu/mL
Wareham <i>et al.</i> (486)	2011/ UK	<i>A. baumannii</i> (6)	'COL' * alone 'COL' + TEC	Five of six isolates were MDR; susceptibility to COL was not reported. Concentrations used were COL 1 mg/L and TEC 20 mg/L. Monotherapy with COL was bactericidal against all isolates at 4 h, with regrowth to control levels of all MDR isolates at 24 h; a reference strain showed no regrowth at this time. Monotherapy with TEC was not performed. With COL + TEC no regrowth occurred and there was a >8-fold log difference in viable counts compared with COL monotherapy at 24 h and a ≥4-fold reduction compared with the starting inoculum.	Initial inoculum: $>1 \times 10^5$ cfu/mL
Sheng <i>et al.</i> (482)	2011/ Taiwan	<i>A. baumannii</i> (12) <i>A. genospecies</i> 13TU (3) <i>A. genospecies</i> 3 (2)	'COL' * alone IMI alone TIG alone 'COL' + IMI (all isolates) 'COL' + TIG (2 isolates)	All isolates were resistant to IMI; COL MICs ranged from 0.125 – 4 mg/L, although it is unclear whether MIC determinations were performed with CMS or COL. All antimicrobial agents used at concentrations of 0.5× MIC. COL + IMI was synergistic at 24 h against 9 (75%) of 12, 3 (100%) of 3 and 2 (100%) of 2 isolates of <i>A. baumannii</i> , <i>A. genospecies</i> 13TU and <i>A. genospecies</i> 3, respectively. COL + TIG was markedly synergistic at 24 h against 2 <i>A. baumannii</i> isolates, with no viable bacteria detected at this time.	Initial inoculum: 5×10^5 cfu/mL Population analysis profiles did not reveal any colistin- or imipenem-heteroresistant isolates.

* Colistin (sulphate) or CMS (sodium) not specified

Table A1-3. (Continued)

Shields <i>et al.</i> (490)	2011/ USA	MDR <i>A. baumannii</i> (5)	'COL' * alone DOR alone 'COL' + DOR	All isolates were considered colistin-susceptible (MICs ≤ 2 mg/L); it is unclear whether MIC determinations were performed with CMS or COL. DOR 8 mg/L showed no activity whereas COL 0.25 mg/L was bacteriostatic. DOR + COL at the same concentrations resulted in killing to below the level of detection by 8 h with no regrowth evident at 24 h.	Initial inoculum: 1×10^6 cfu/mL Based on <i>in vitro</i> data obtained in this study combinations of DOR (500 mg 8-hourly) and CMS (5 mg/kg/day in 2 – 4 divided doses) were used in patients who received solid organ transplants and were infected with MDR <i>A. baumannii</i> . To date four patients have been treated with this combination, and a fifth patient received meropenem plus colistin. Four (80%) of the 5 patients had a positive clinical response and survived.
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* Colistin (sulphate) or CMS (sodium) not specified

AMI, amikacin; AMP/SUL, ampicillin/sulbactam; CIP, ciprofloxacin; CMS, colistin methanesulphonate (sodium); COL, colistin (sulphate); CTZ, ceftazidime; DOR, doripenem; IMI, imipenem; MER, meropenem; MIN, minocycline; PB, polymyxin B; RIF, rifampicin; TEC, teicoplanin; TIG, tigecycline; TOB, tobramycin; TRI/SUL, trimethoprim/sulfamethoxazole; VAN, vancomycin; cfu, colony forming units; MBL, metallo- β -lactamase; MDR, multidrug-resistant; MIC, minimum inhibitory concentration; PK/PD, pharmacokinetic/pharmacodynamic.

Appendix 2
Additional time-kill and log change data for Chapter 5

Table A2-1: Log changes in viable cell counts at 6, 24, and 48 h with various clinically relevant concentrations of colistin (Col) and imipenem (Imi) against three *P. aeruginosa* isolates susceptible to both antibiotics. A gray background indicates activity (a reduction of ≥ 1 log₁₀ cfu/mL below the initial inoculum); a green background indicates synergy (a ≥ 2 -log₁₀ decrease in the number of cfu/mL with the combination from that with its most active component); and a red background indicates additivity (a 1.0- to < 2 -log₁₀ decrease in the number of cfu/mL with the combination from that with its most active component).

Isolate	Inoculum (cfu/mL)	Time (h)	Log change (= log ₁₀ (CFU _t) - log ₁₀ (CFU ₀))														
			Col 0.5× MIC	Col 4× MIC	Col 16× MIC*	Imi 0.5× MIC	Imi 4× MIC	Imi 16× MIC	Col 0.5× MIC +	Col 0.5× MIC +	Col 0.5× MIC +	Col 4× MIC +	Col 4× MIC +	Col 4× MIC +	Col 16× MIC* +	Col 16× MIC* +	Col 16× MIC* +
ATCC 27853	~10 ⁶	6	-0.41	-5.93	-6.03	-0.03	-2.77	-2.83	-2.90	-4.39	-5.95	-4.69	-5.95	-5.99	-5.98	-5.97	-6.01
		24	+3.20	+0.06	-1.23	+2.91	-3.14	-3.66	+1.71	-3.58	-5.95	-2.20	-2.68	-3.34	-0.35	-1.65	-6.01
		48	+3.80	+1.35	+0.39	+3.60	-1.06	-1.81	+3.49	-2.39	-2.90	+0.04	-1.82	-2.27	+0.90	-0.98	-6.01
	~10 ⁸	6	+0.33	-2.48	-7.68	-0.06	-2.04	-2.08	-1.00	-2.79	-2.73	-5.62	-4.94	-4.69	-7.76	-7.78	-7.81
		24	+1.55	+0.05	-3.34	+1.67	+0.14	-3.73	+1.50	-0.48	-3.42	-0.01	-3.51	-4.54	-2.83	-7.78	-7.81
		48	+2.05	+1.65	-1.97	+2.02	+1.96	-2.89	+2.04	+1.79	-3.30	+0.92	-2.25	-3.60	-1.46	-7.78	-7.81

Table A2-1. (Continued)

Isolate	Inoculum (cfu/mL)	Time (h)	Log change (= $\log_{10}(\text{CFU}_t) - \log_{10}(\text{CFU}_0)$)															
			Col 0.5× MIC	Col 4× MIC	Col 16× MIC*	Imi 0.5× MIC	Imi 4× MIC	Imi 16× MIC	Col 0.5× MIC +	Col 0.5× MIC +	Col 0.5× MIC +	Col 4× MIC +	Col 4× MIC +	Col 4× MIC +	Col 16× MIC +	Col 16× MIC +	Col 16× MIC +	
19056 muc	~10 ⁶	6	-2.34	-5.19	-5.16	+0.45	-3.81	-5.49	-5.66	-5.69	-5.79	-5.88	-5.92	-5.75	-5.82	-5.89	-5.88	
		24	+1.63	-2.64	-5.16	+2.66	-3.41	-5.49	+1.69	-4.39	-5.79	-2.62	-5.92	-5.75	-5.82	-5.89	-5.88	
		48	+3.13	+0.08	-0.37	+3.27	+1.93	-5.49	+2.53	-0.27	-5.79	-0.44	-1.06	-5.75	-5.82	-5.89	-5.88	
	~10 ⁸	6	+0.11	-7.51	-7.94	-0.83	-3.76	-4.22	-1.68	-4.00	-4.05	-7.96	-8.22	-7.95	-7.94	-7.91	-7.76	
		24	+0.79	-3.49	-7.94	-0.01	-3.22	-5.02	+0.19	-3.46	-4.60	-2.50	-6.92	-7.95	-7.94	-7.91	-7.76	
		48	+1.42	-0.15	-5.03	+0.47	+0.24	-6.08	+0.39	+0.27	-5.91	+0.08	-3.18	-7.95	-4.00	-4.45	-7.76	
	20509 n/m	~10 ⁶	6	+1.47	-3.18	-5.89	-1.71	-3.08	-3.83	-2.29	-5.97	-6.14	-4.34	-6.10	-5.90	-5.95	-6.05	-6.05
			24	+3.18	+2.39	-0.94	+3.10	-2.30	-3.33	+3.26	-3.04	-3.93	+1.86	-4.41	-4.30	-3.35	-6.05	-6.05
			48	+3.61	+3.07	+0.81	+3.46	-1.08	-1.43	+3.48	-1.00	-1.68	+3.21	-1.40	-1.93	-1.80	-4.27	-3.58
~10 ⁸		6	+0.82	-0.01	-3.41	-0.81	-2.65	-2.65	-0.52	-2.86	-2.55	-1.03	-2.75	-2.53	-3.50	-4.94	-7.94	
		24	+1.62	+2.04	-0.28	+1.02	-0.45	-1.48	+1.40	-0.59	-2.60	+0.81	-1.88	-3.63	-1.58	-3.71	-4.49	
		48	+1.92	+2.19	+1.89	+1.25	+0.96	-1.28	+1.70	+1.58	-0.98	+1.75	+1.03	-2.37	+1.03	-1.42	-3.22	

*For ATCC 27853, Col 8× MIC used instead of 16× MIC at the 10⁶ cfu/mL inoculum only.

Table A2-2. Log changes in viable cell counts at 6, 24, and 48 h with various clinically relevant concentrations of colistin (Col) and imipenem (Imi) against one colistin-resistant, imipenem-susceptible isolate and two colistin-susceptible, imipenem-resistant isolates of *P. aeruginosa*. A gray background indicates activity (a reduction of $\geq 1 \log_{10}$ cfu/mL below the initial inoculum); a green background indicates synergy (a $\geq 2\text{-log}_{10}$ decrease in the number of cfu/mL with the combination from that with its most active component); and a red background indicates additivity (a 1.0- to $<2\text{-log}_{10}$ decrease in the number of cfu/mL with the combination from that with its most active component).

Isolate	Inoculum (cfu/mL)	Time (h)	Log change (= $\log_{10}(\text{CFU}_t) - \log_{10}(\text{CFU}_0)$)													
			Col 32 mg/L	Imi 0.5× MIC	Imi 4× MIC	Imi 16× MIC	Col 1.0 mg/L	Col 1.0 mg/L	Col 1.0 mg/L	Col 4.0 mg/L	Col 4.0 mg/L	Col 4.0 mg/L	Col 32 mg/L	Col 32 mg/L	Col 32 mg/L	
Col- resistant, Imi- susceptible							+	+	+	+	+	+	+	+		
							Imi 0.5× MIC	Imi 4× MIC	Imi 16× MIC	Imi 0.5× MIC	Imi 4× MIC	Imi 16× MIC	Imi 0.5× MIC	Imi 4× MIC	Imi 16× MIC	
	19147	$\sim 10^6$	6	-0.08	-0.35	-1.44	-1.28	-1.28	-1.46	-1.77	-1.77	-1.60	-1.83	-2.81	-2.79	-3.15
	n/m ^a		24	+2.16	+2.27	-0.58	-2.57	-0.71	-3.28	-4.57	-2.01	-3.89	-4.22	-1.95	-3.95	-4.26
			48	+2.49	+3.02	+2.50	-4.55	+2.57	-3.03	-4.46	+1.74	-1.99	-3.54	+1.58	-3.72	-4.21
		$\sim 10^8$	6	+0.04	-1.04	-1.56	-1.36	-1.83	-1.82	-1.85	-2.40	-2.70	-2.54	-2.54	-2.65	-2.79
		24	+0.55	+1.31	-0.33	-2.67	+0.14	-3.24	-3.38	-1.10	-4.60	-4.61	-1.02	-5.90	-6.11	
		48	+0.86	+1.79	+1.37	-3.21	+1.18	-0.10	-3.19	+0.42	-3.82	-7.69	-0.02	-3.09	-7.71	

Table A2-2. (Continued)

Isolate	Inoculum (cfu/mL)	Time (h)	Log change (= log ₁₀ (CFU _t) - log ₁₀ (CFU ₀))															
			Col 0.5× MIC	Col 4× MIC	Col 16× MIC*	Imi 1.0 mg/L	Imi 8.0 mg/L	Imi 32 mg/L	Col 0.5× MIC	Col 0.5× MIC	Col 0.5× MIC	Col 4× MIC	Col 4× MIC	Col 4× MIC	Col 16× MIC	Col 16× MIC	Col 16× MIC	
Col- susceptible, Imi- resistant	~10 ⁶	6	-1.89	-3.32	-3.88	+1.68	+1.17	-0.54	-1.32	-2.00	-4.45	-3.43	-5.77	-5.71	-5.70	-5.77	-5.75	
		24	+0.49	-2.19	-3.68	+2.95	+2.89	-1.27	+0.71	+0.24	-5.75	-3.00	-5.77	-5.71	-3.92	-5.77	-5.75	
		48	+2.87	+1.83	-1.29	+3.01	+2.93	+1.56	+2.88	+2.81	-2.61	+0.15	-1.52	-5.71	-2.89	-5.77	-5.75	
		~10 ⁸	6	-0.05	-2.22	-3.18	+0.56	+0.26	-0.95	-1.05	-0.94	-1.57	-3.13	-3.48	-4.90	-4.81	-4.97	-5.36
		24	+0.67	-1.13	-2.95	+0.84	+0.39	-1.70	-0.37	-0.08	-2.47	-1.86	-1.76	-3.88	-2.52	-3.66	-6.22	
		48	+0.83	-1.26	-0.46	+0.83	+0.46	+0.15	+0.94	+0.98	+0.25	-1.09	+0.22	-2.30	-0.01	-3.14	-4.24	
	20891 n/m	~10 ⁶	6	+1.38	-5.95	-5.97	+1.68	-0.26	-2.61	+0.35	-6.02	-5.90	-4.62	-5.90	-5.83	-5.92	-5.96	-5.98
			24	+2.28	-3.43	-4.57	+2.53	+2.27	-3.82	+2.35	-2.90	-5.90	-3.85	-3.64	-5.83	-5.92	-5.96	-5.98
			48	+2.48	-1.84	-3.20	+2.42	+2.28	+0.01	+2.43	+2.44	-3.82	-0.42	-3.34	-5.83	-3.77	-5.96	-5.98
		~10 ⁸	6	+0.21	-4.67	-5.50	+0.13	-0.77	-2.50	-0.33	-1.32	-2.54	-5.76	-7.39	-7.52	-5.97	-7.35	-7.52
			24	+0.60	-1.12	-2.65	+0.32	-0.31	-3.64	+0.54	+0.47	-2.38	-3.39	-3.47	-4.27	-4.60	-5.35	-4.83
			48	+0.77	-0.69	-2.61	+0.55	+0.21	+0.07	+0.91	+0.84	+1.00	-3.08	-2.44	-2.89	-3.65	-3.92	-3.58

^a For colistin-resistant isolate 19147 n/m, synergy or additivity were compared with imipenem monotherapy only.

Time-kill plots:

Figure A2-1. Time-kill curves for ATCC 27853 at an inoculum of $\sim 10^6$ cfu/mL with (A) colistin alone, (B) imipenem alone, and (C) in combination. Panel (D) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and LOQ is indicated by the dashed horizontal line.

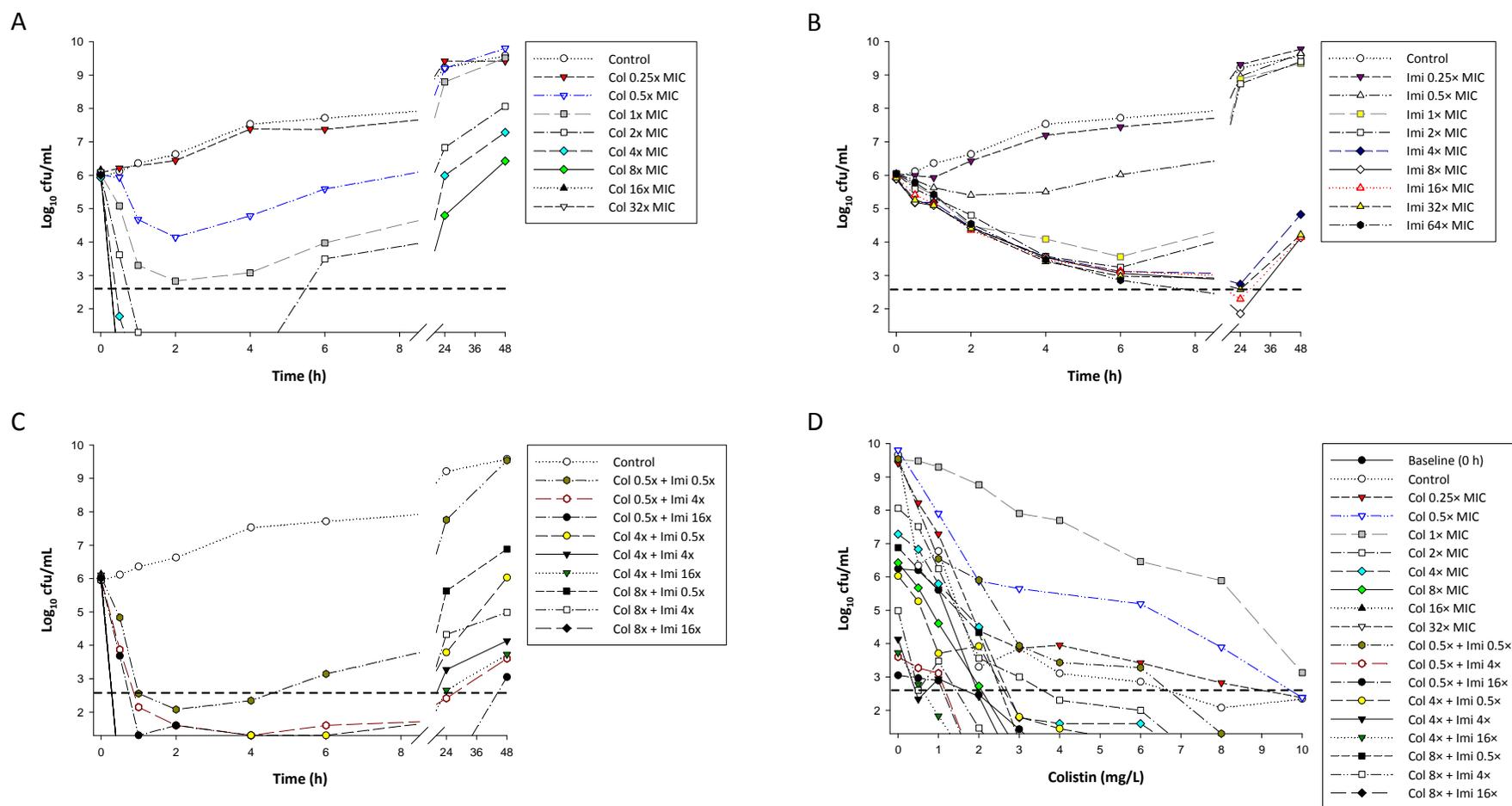


Figure A2-2. Time-kill curves for ATCC 27853 at an inoculum of $\sim 10^8$ cfu/mL with (A) colistin alone, (B) imipenem alone, and (C) in combination. Panel (D) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the LOQ is indicated by the dashed horizontal line.

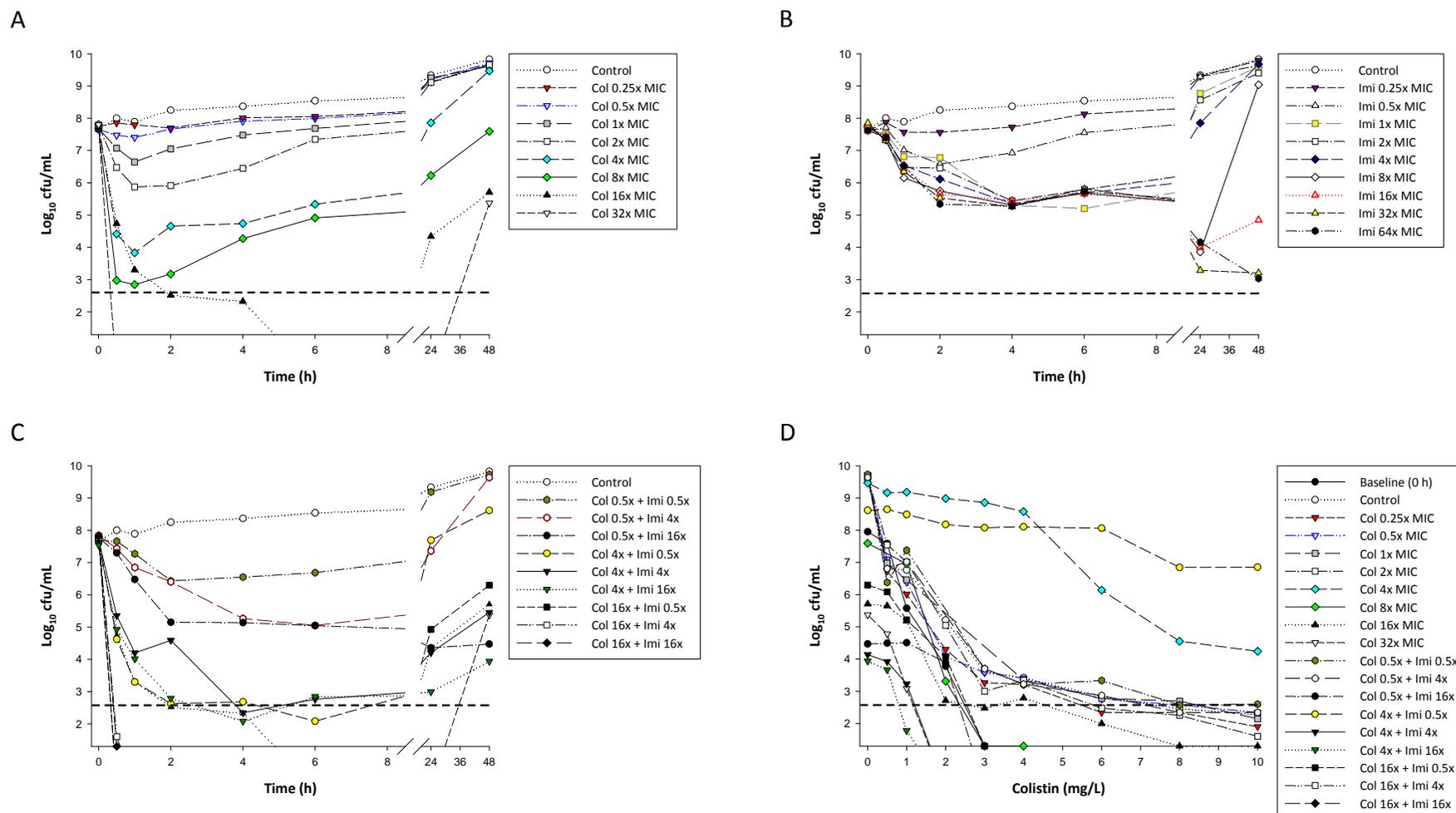


Figure A2-3. Time-kill curves for 19147 n/m at an inoculum of $\sim 10^6$ cfu/mL with (A) colistin and imipenem alone, and (B) in combination. Panel (C) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the LOQ is indicated by the dashed horizontal line.

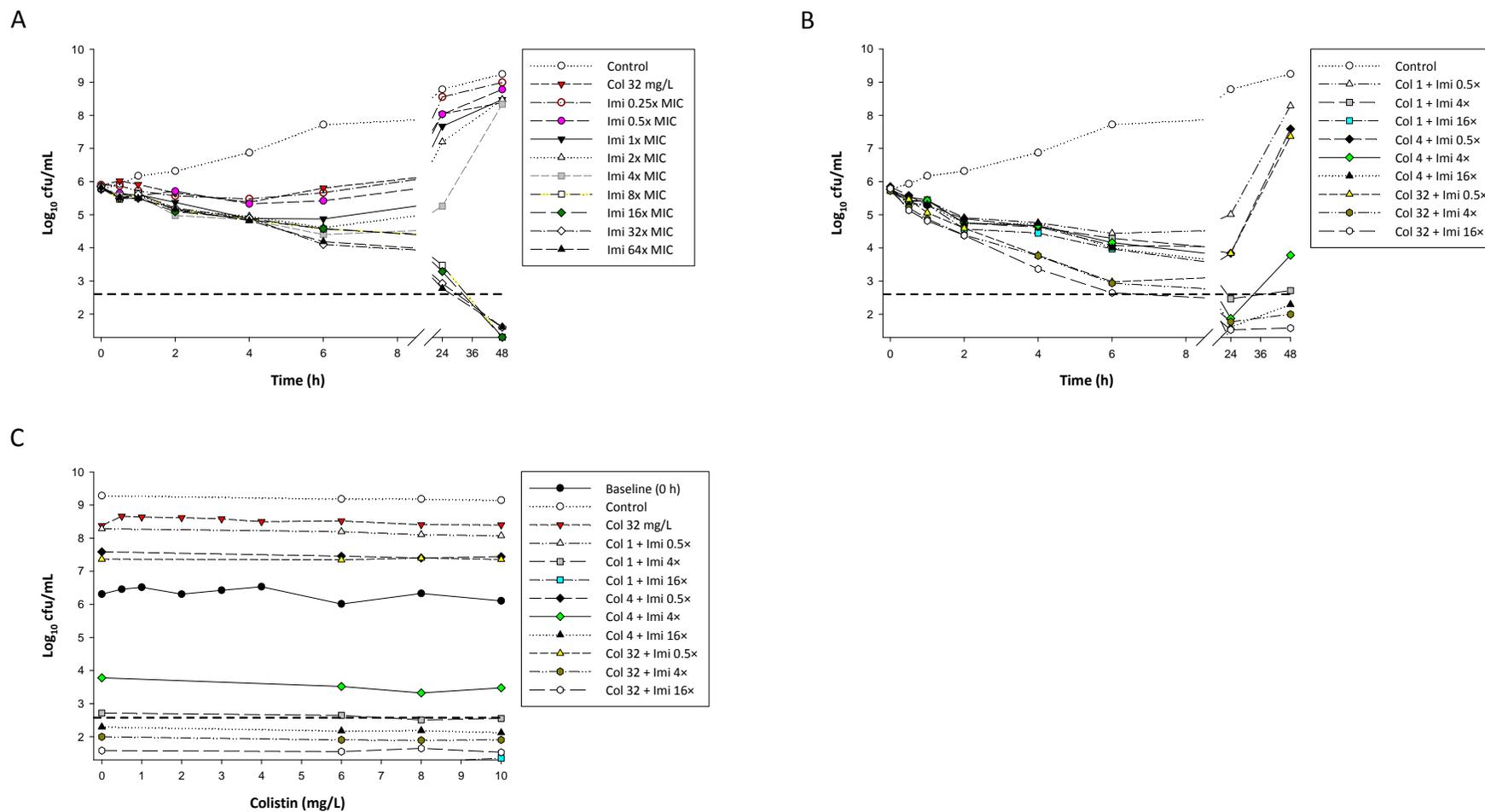


Figure A2-4. Time-kill curves for 19147 n/m at an inoculum of $\sim 10^8$ cfu/mL with (A) colistin and imipenem alone, and (B) in combination. Panel (C) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the LOQ is indicated by the dashed horizontal line.

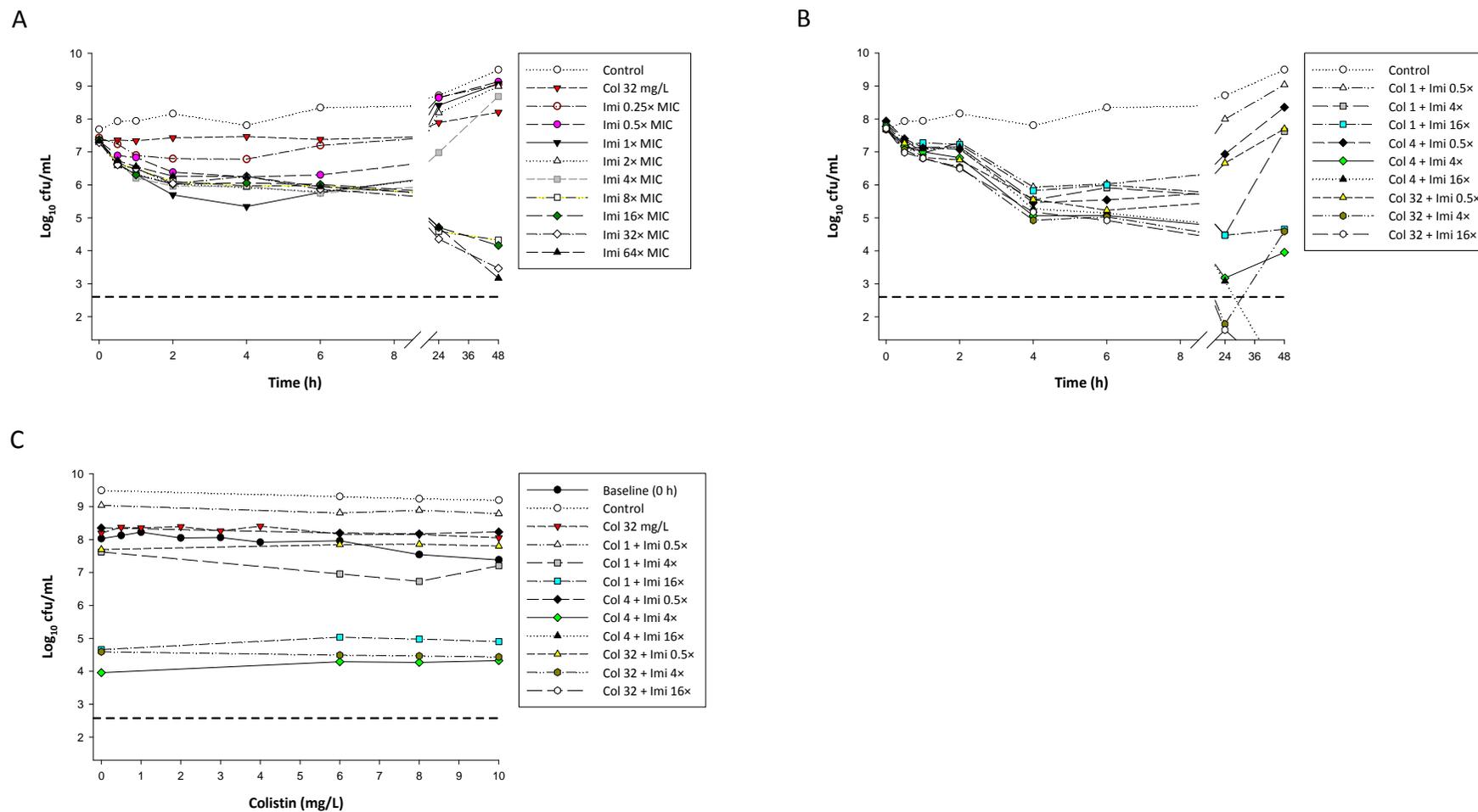


Figure A2-5. Time-kill curves for 19056 muc at an inoculum of $\sim 10^6$ cfu/mL with (A) colistin alone, (B) imipenem alone, and (C) in combination. Panel (D) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the LOQ is indicated by the dashed horizontal line.

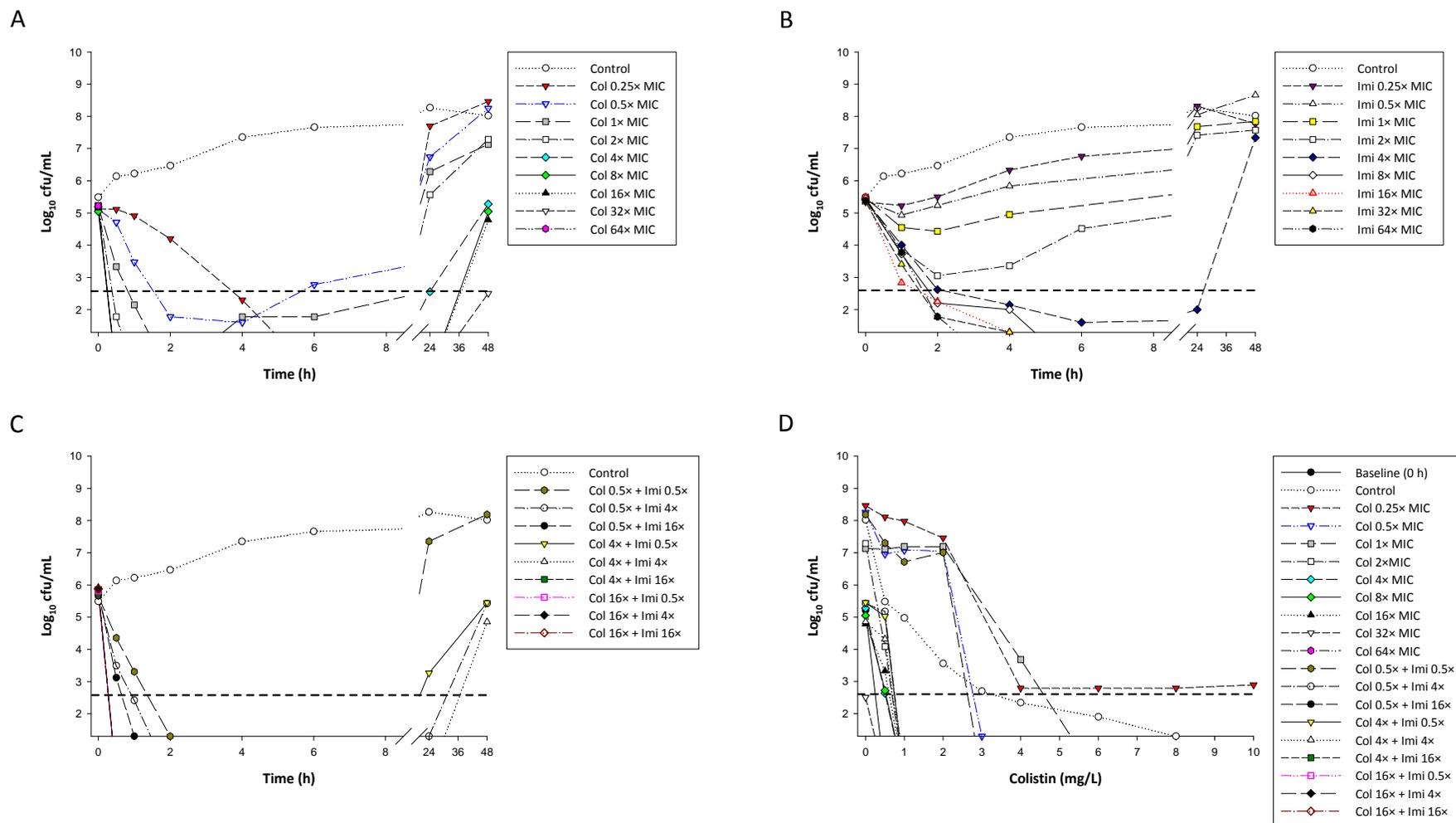


Figure A2-6. Time-kill curves for 19056 muc at an inoculum of $\sim 10^8$ cfu/mL with (A) colistin alone, (B) imipenem alone, and (C) in combination. Panel (D) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the LOQ is indicated by the dashed horizontal line.

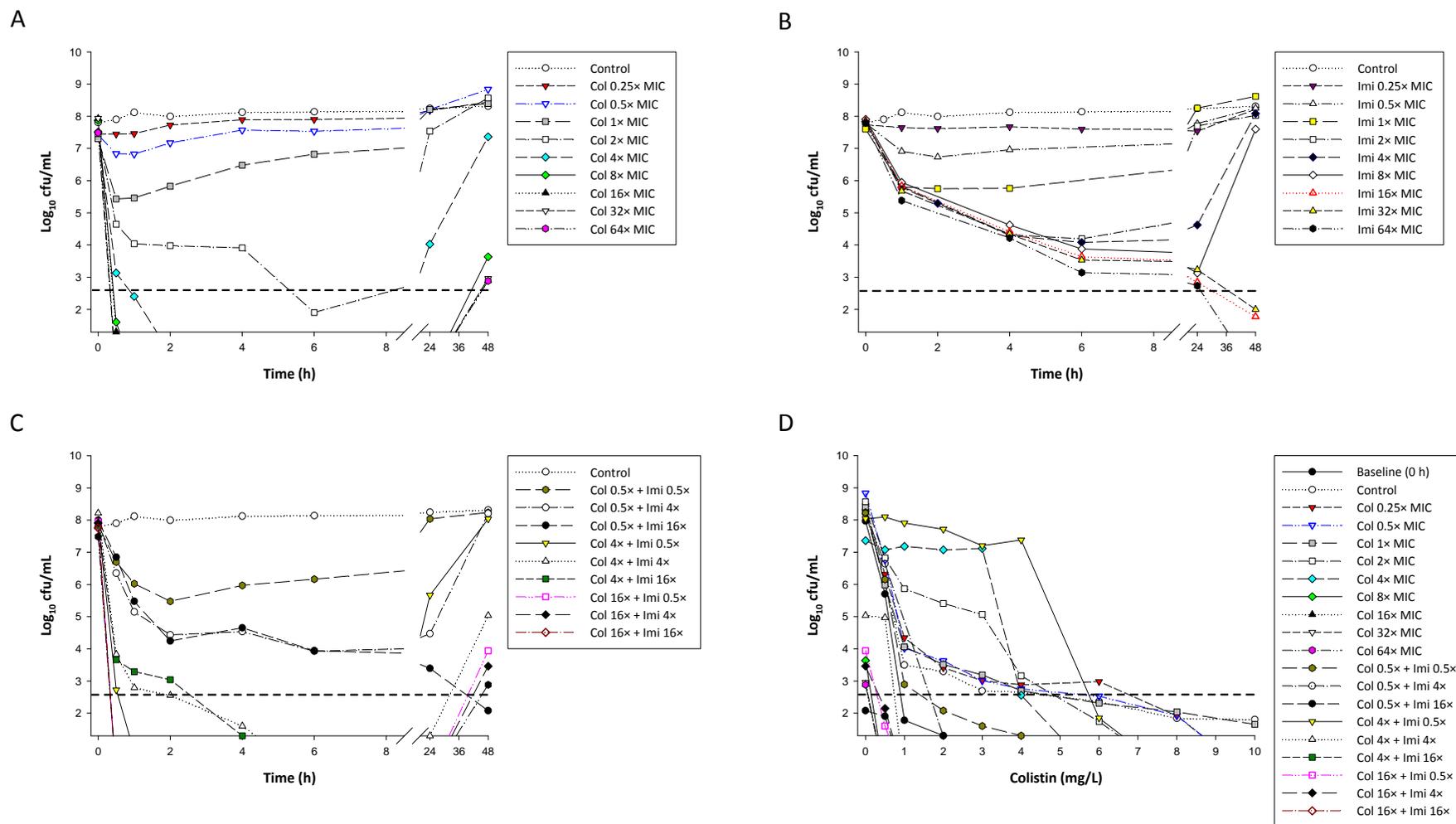


Figure A2-7. Time-kill curves for 20509 n/m at an inoculum of $\sim 10^6$ cfu/mL with (A) colistin alone, (B) imipenem alone, and (C) in combination. Panel (D) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the LOQ is indicated by the dashed horizontal line.

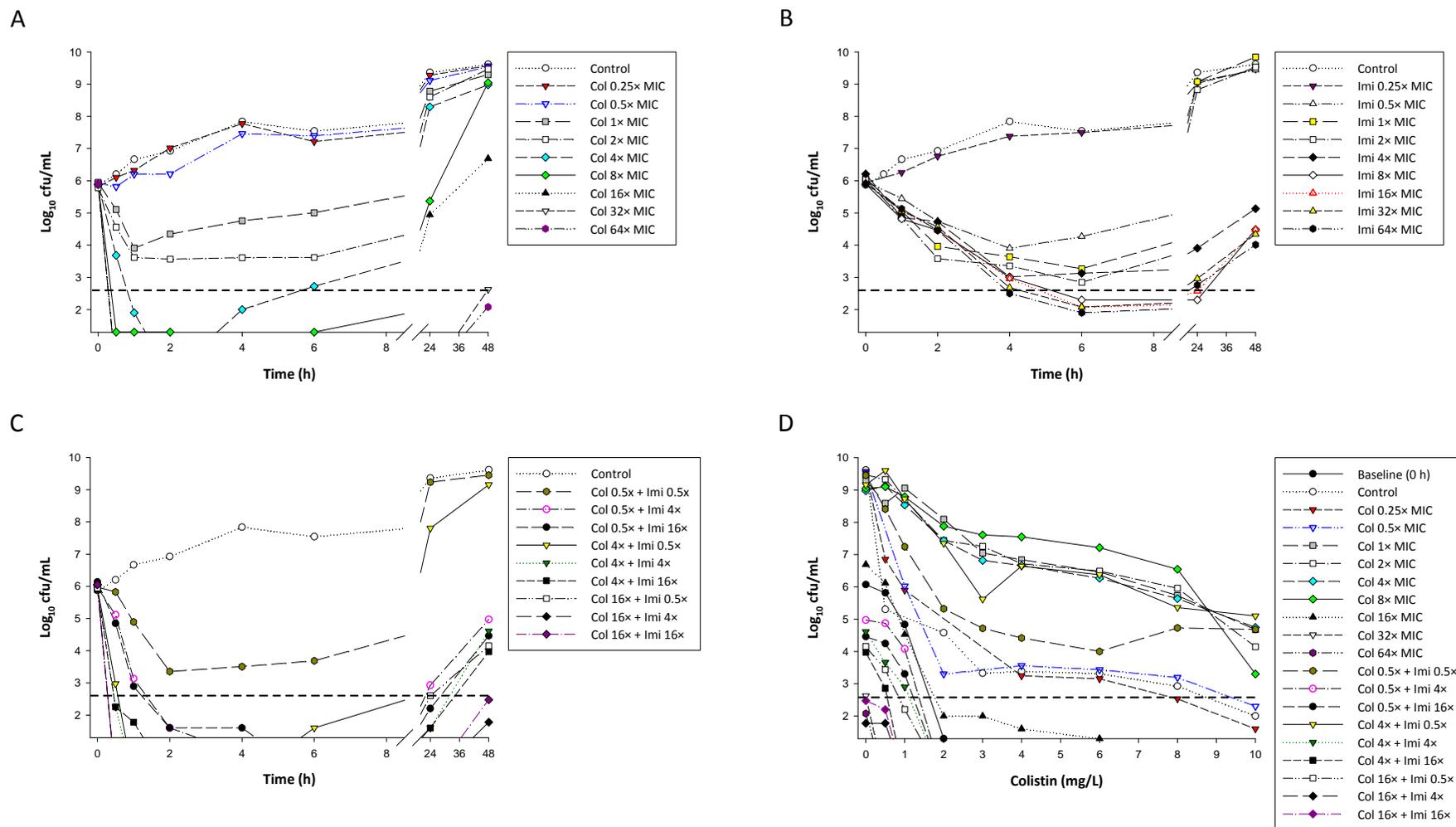


Figure A2-8. Time-kill curves for 20509 n/m at an inoculum of $\sim 10^8$ cfu/mL with (A) colistin alone, (B) imipenem alone, and (C) in combination. Panel (D) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the LOQ is indicated by the dashed horizontal line.

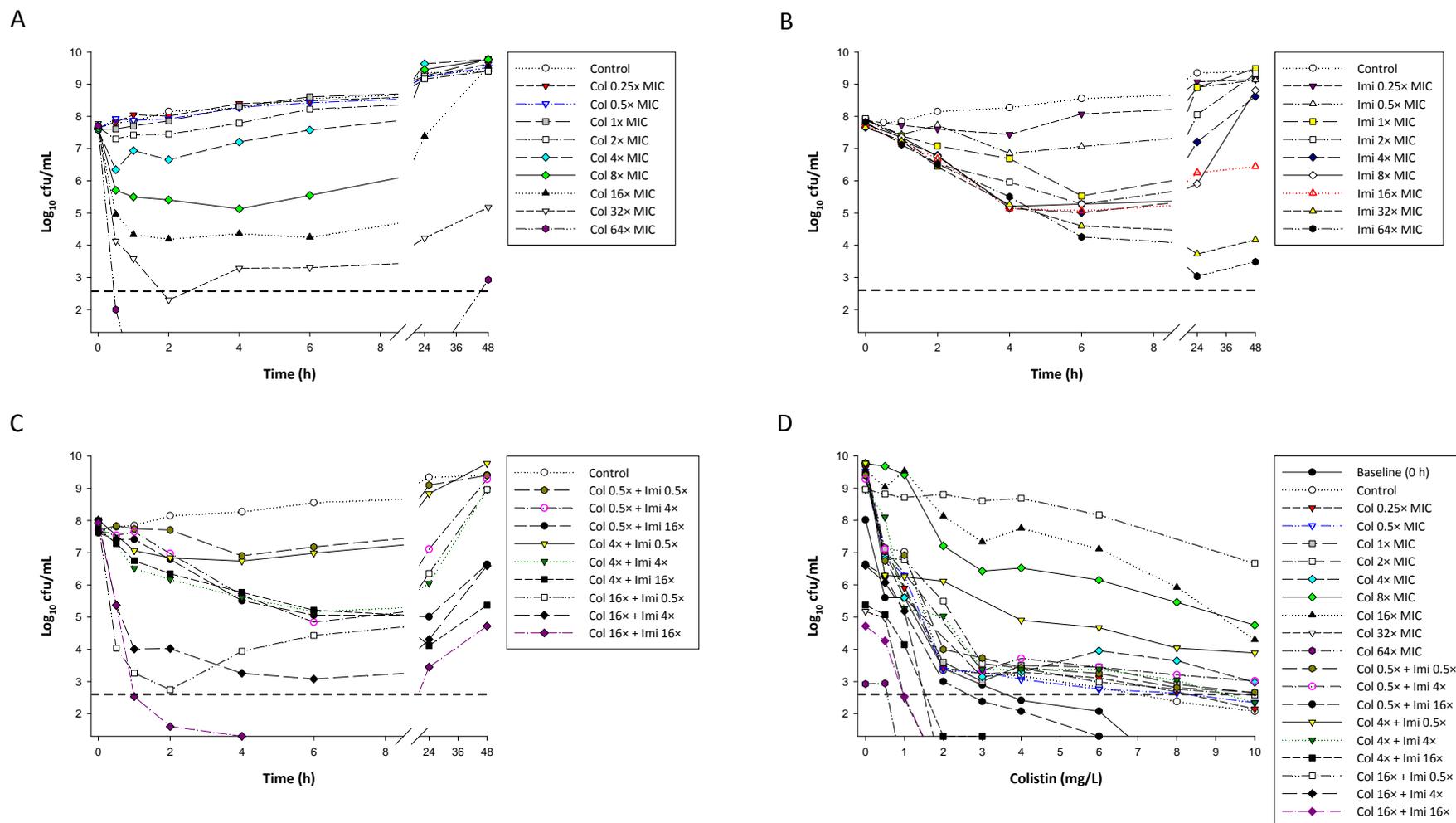


Figure A2-9. Time-kill curves for 19271 n/m at an inoculum of $\sim 10^6$ cfu/mL with (A) colistin and imipenem alone, and (B) in combination. Panel (C) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the LOQ is indicated by the dashed horizontal line.

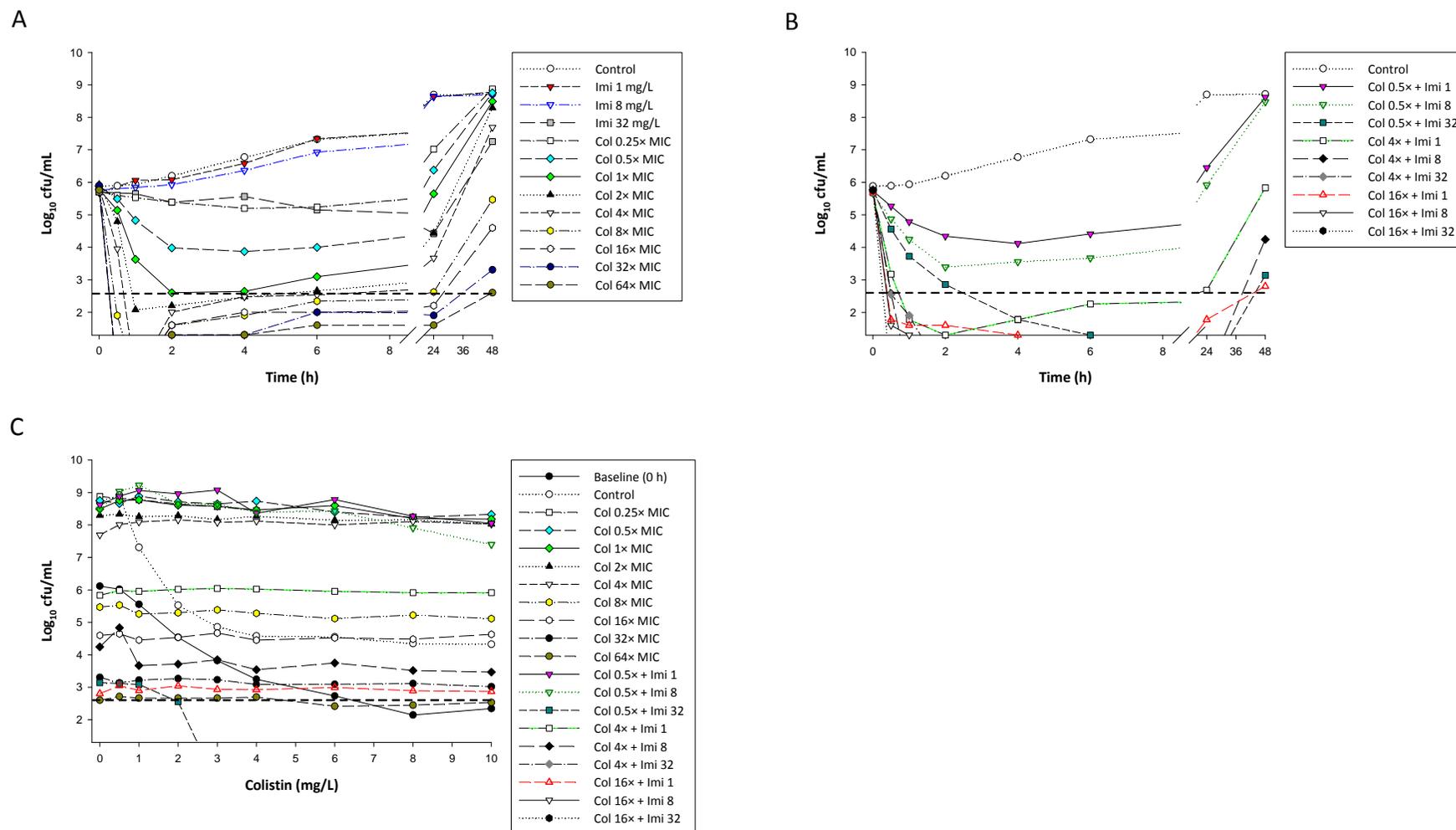


Figure A2-10. Time-kill curves for 19271 n/m at an inoculum of $\sim 10^8$ cfu/mL with (A) colistin and imipenem alone, and (B) in combination. Panel (C) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the LOQ is indicated by the dashed horizontal line.

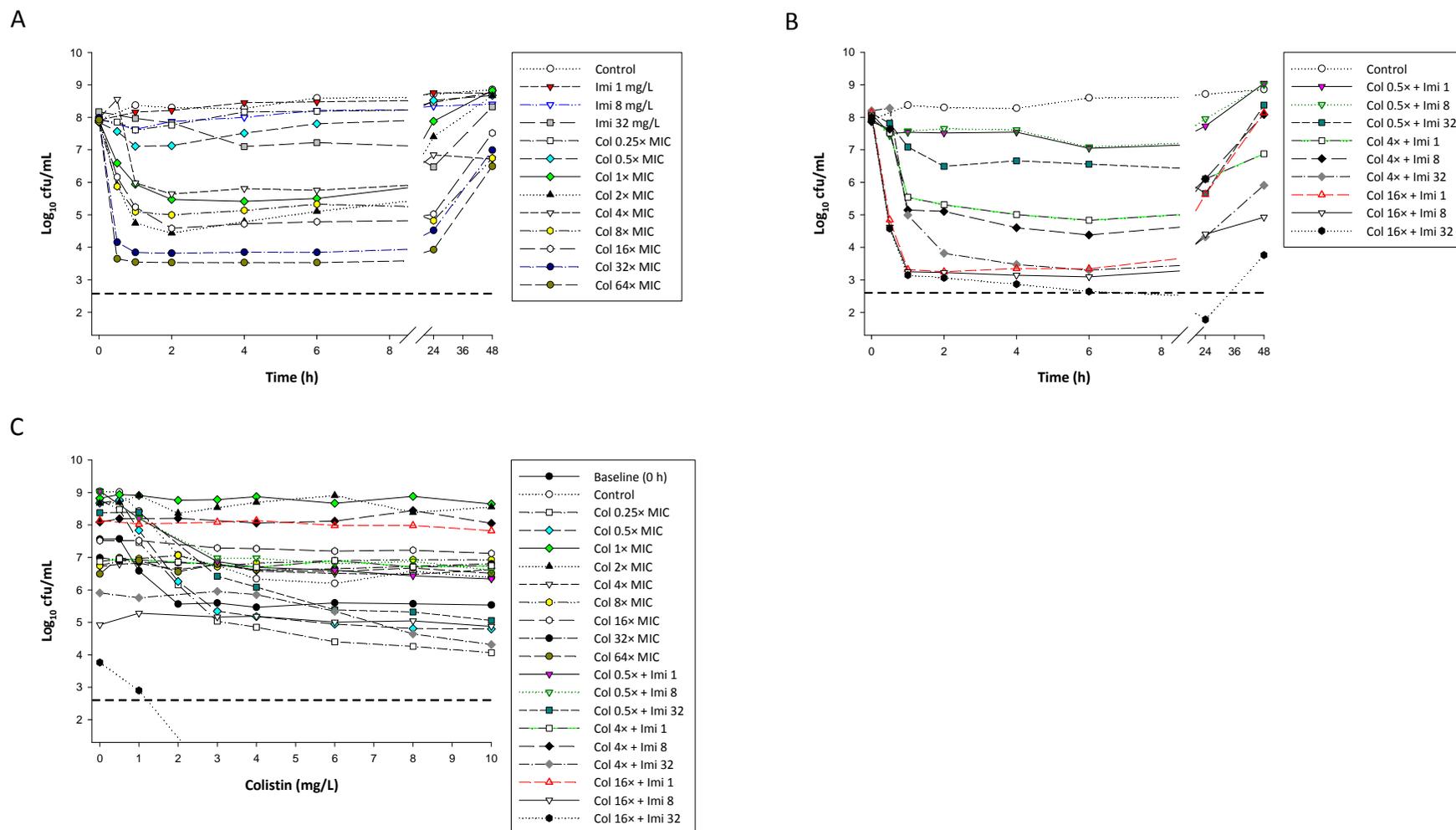


Figure A2-11. Time-kill curves for 20891 n/m at an inoculum of $\sim 10^6$ cfu/mL with (A) colistin and imipenem alone, and (B) in combination. Panel (C) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the LOQ is indicated by the dashed horizontal line.

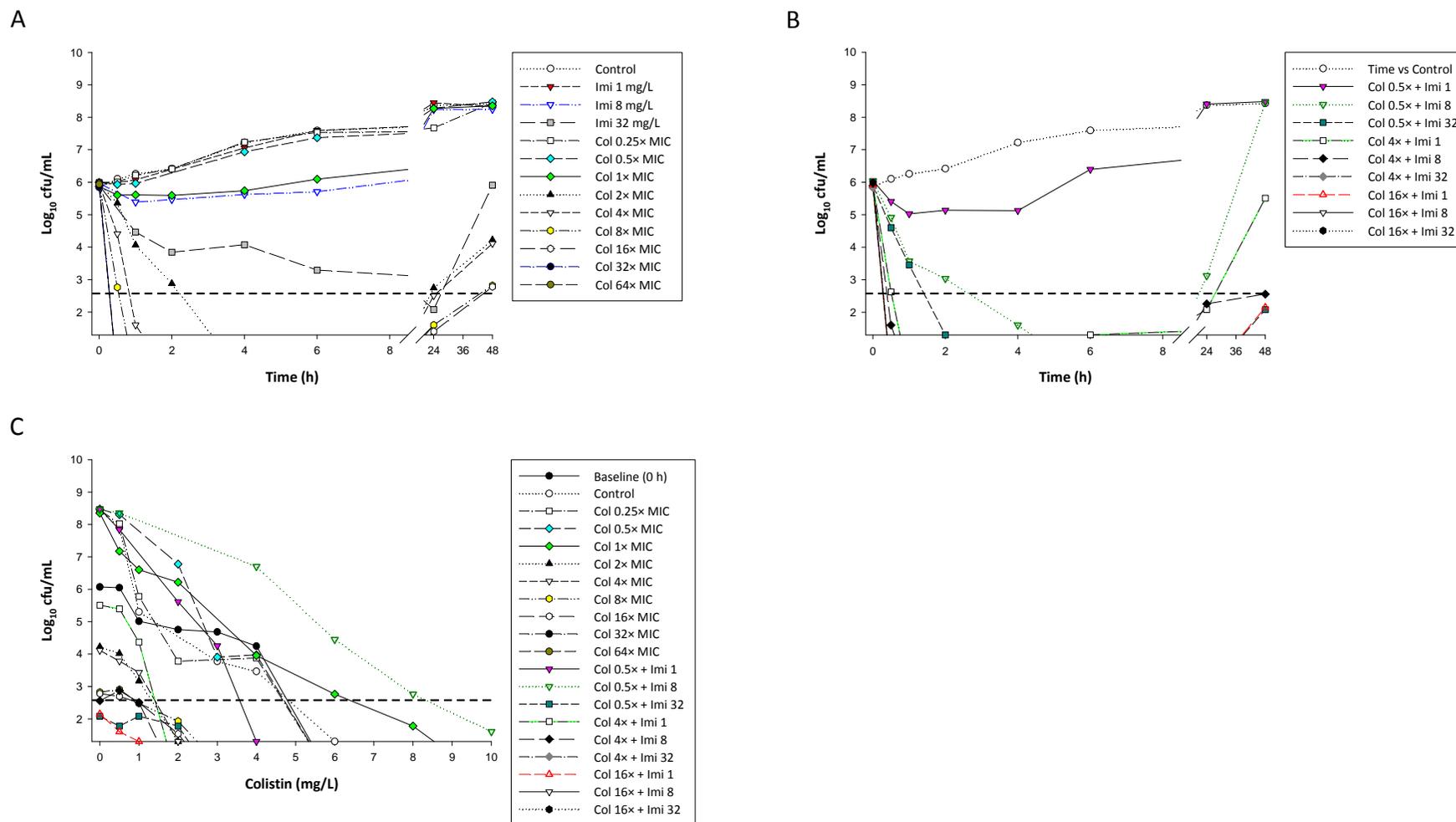
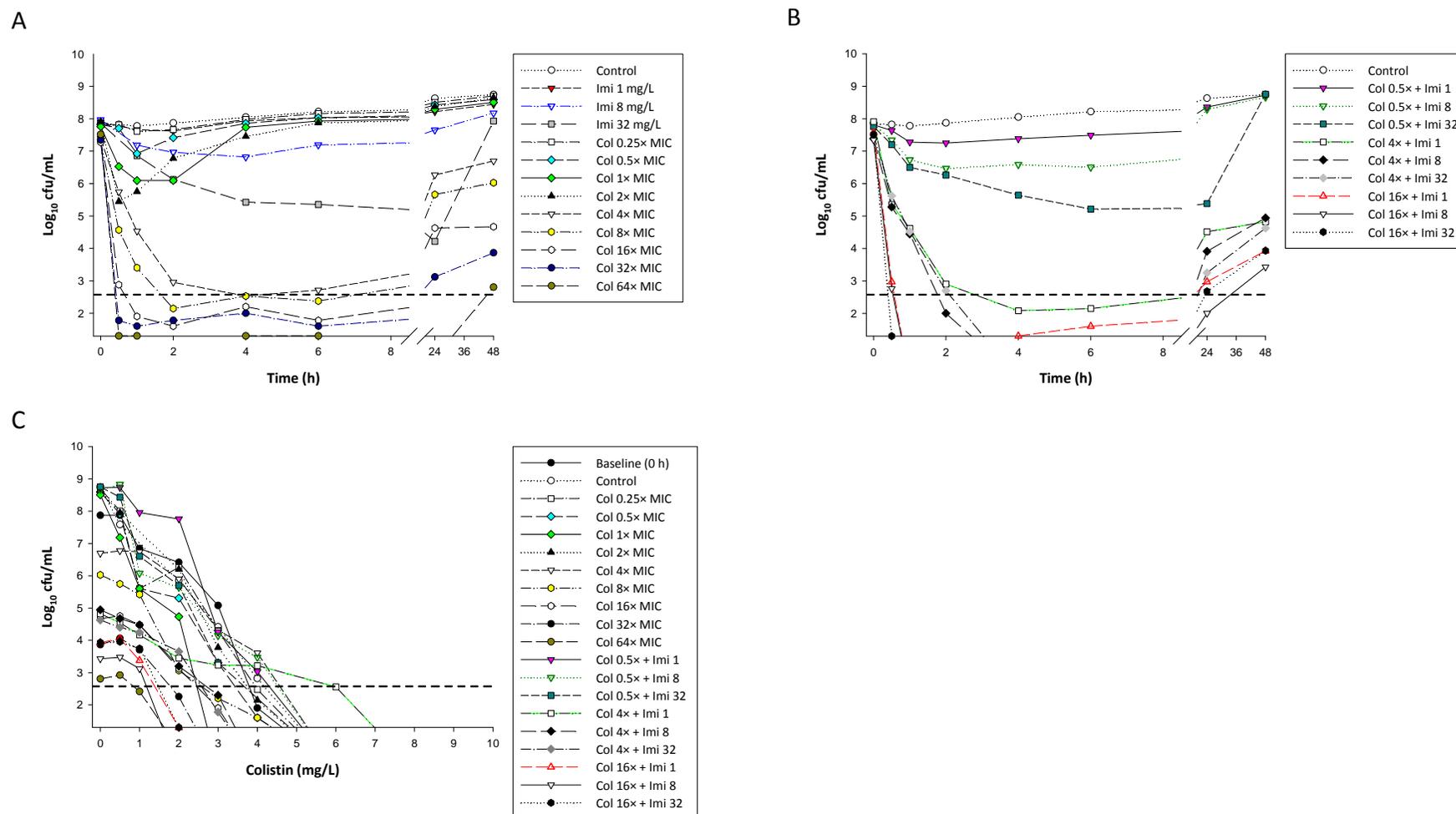


Figure A2-12. Time-kill curves for 20891 n/m at an inoculum of $\sim 10^8$ cfu/mL with (A) colistin and imipenem alone, and (B) in combination. Panel (C) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the LOQ is indicated by the dashed horizontal line.



Appendix 3
Publications in support of this thesis



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Dosing of colistin—back to basic PK/PD

Phillip J Bergen, Jian Li^a and Roger L Nation^a

The increasing prevalence of multidrug-resistant Gram-negative bacteria worldwide has led to a re-evaluation of the previously discarded antibiotic, colistin. Despite its important role as salvage therapy for otherwise untreatable infections, dosage guidelines for the prodrug colistin methanesulfonate (CMS) are not scientifically based and have led to treatment failure and increased colistin resistance. In this review we summarise the recent progress made in the understanding of the pharmacokinetics of CMS and formed colistin with an emphasis on critically ill patients. The pharmacodynamics of colistin is also reviewed, with special attention given to the relationship between pharmacokinetics and pharmacodynamics and how the emerging data can be used to inform design of optimal dosage regimens. Recent data suggest the current dosage regimens of CMS are suboptimal in many critically ill patients.

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Introduction

Several highly resistant Gram-negative bacteria – namely *Acinetobacter* species, *Pseudomonas aeruginosa* and carbapenem-resistant *Klebsiella* species – are emerging as significant pathogens worldwide [1]. Therapeutic options for these pathogens are extremely limited, a situation made worse by the drying up of the pharmaceutical development pipeline for anti-infective agents [2]. This has forced clinicians to return to using older, previously discarded drugs, such as the polymyxins [1,3,4^{••}]. Of the two polymyxins used clinically (polymyxin B and E), the latter also known as colistin is used most widely and is the subject of this review. Having entered clinical use in 1959, colistin was never subjected to the battery of drug development procedures now mandated by international drug regulatory authorities. The result was a

dearth of pharmacological information to inform rational use in order to maximize antibacterial activity and minimize toxicity and development of resistance [5]. Although colistin currently retains significant *in vitro* activity against many isolates of the above-mentioned Gram-negative pathogens, resistance to this crucial last-line therapy is emerging [6–8].

Colistin is a cationic antimicrobial peptide. As a detailed review of the chemistry of colistin is beyond the scope of this article, we refer interested readers elsewhere [5,9]. Given the relatively high level of toxicity associated with parenteral administration of colistin (sulfate) in early studies, a less toxic sulfomethyl derivative, colistin methanesulfonate (CMS), was developed [10]. Although CMS is the form administered parenterally, CMS undergoes conversion *in vivo* to colistin as discussed below. Importantly, it is this formed colistin that is responsible for antibacterial activity, not CMS, and thus CMS should be considered an inactive prodrug [11[•]]. The active species, colistin, has a narrow antibacterial spectrum mainly against common Gram-negative bacteria including *P. aeruginosa*, *Acinetobacter* spp. and *Klebsiella* spp. [12]. It is against these organisms that colistin is most commonly used clinically, particularly in critically ill patients [1,5].

Recent investigations have unraveled key aspects of the pharmacokinetics (PK) and pharmacodynamics (PD) of colistin, and these aspects are the focus of this brief review.

Pharmacokinetics of CMS and formed colistin

Despite its availability for over 50 years, it is only with the development of HPLC and LC/MS/MS analytical methods in the past 5–10 years that an accurate picture of the PK of CMS and formed colistin has emerged. The demonstration that antimicrobial activity results from colistin, formed *in vivo* following administration of CMS, made the separate quantification of the inactive prodrug (CMS) and active entity (colistin) a prerequisite for accurate PK information. Before the development of these techniques, microbiological assays incapable of separately quantifying CMS and colistin were employed for measurement of ‘colistin’ concentrations. Importantly, the PK and prescribing information supplied with currently available parenteral products was obtained using microbiological assays.

All CMS/colistin PK data discussed below were obtained using HPLC or LC/MS/MS analytical methods. This section will focus mainly on preclinical PK studies that have involved separate administration of CMS and

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colistin and that have revealed key aspects of the disposition of not only the prodrug but also the active entity (colistin) formed from it *in vivo*. Clinical PK will be reviewed in a later section.

Following parenteral administration of CMS, the overall disposition of formed colistin is rate limited by its elimination rather than its formation as indicated by the substantially longer terminal half-life of formed colistin compared with that of the administered CMS, a finding common to both preclinical [13,14[•]] and clinical [15,16,17^{••}] studies. CMS appears to be a relatively inefficient prodrug; in rats, only a very small proportion (~7–16%) of the administered dose of CMS appears to be converted systemically to colistin [13,14[•]]. The PK of CMS and formed colistin appear linear following IV administration of CMS to rats over a range of doses generating clinically relevant plasma concentrations [14[•]]. In pivotal studies conducted in rats, Li *et al.* demonstrated that CMS is predominantly renally cleared (~60% of the dose) with a component of tubular secretion [13], whereas colistin is almost exclusively non-renally cleared (<1% of the dose excreted in urine) and the very low renal clearance involves very extensive renal tubular reabsorption that appears to be carrier mediated [18]. The low *in vivo* conversion of CMS to colistin occurs because the formation clearance of colistin is substantially less than the renal clearance of CMS [13]. Thus, the overall disposition of CMS and formed colistin is very complex. It seems very likely that the renal handling of CMS (net secretion, with the possibility of conversion of CMS to colistin within tubular cells [13]) and formed colistin (avid tubular reabsorption [18,19]), processes that serve to traffic CMS/colistin through tubular cells, may be related to the propensity for CMS therapy to cause nephrotoxicity.

Pharmacodynamics of colistin

While many early reports on antibacterial activity examined both colistin and its parenteral form, CMS, it is important to note that CMS is an inactive prodrug of colistin [11[•]]. Thus, activity reported with the use of CMS, whether from studies conducted *in vitro* or *in vivo*, derives from the formation of the active species, colistin. Polymyxins show excellent *in vitro* activity against ~97% of isolates of *P. aeruginosa* and *Acinetobacter* spp. (MIC₅₀, ≤1 mg/L and MIC₉₀, 2 mg/L for both pathogens) [20]. The majority of PD data on colistin has been generated using static time-kill studies and *in vitro* models. Colistin displays concentration-dependent killing against susceptible strains of *P. aeruginosa*, *A. baumannii* and *K. pneumoniae*, including MDR strains [21–24]. Colistin concentrations in the vicinity of MICs or above result in extremely rapid initial killing, with large decreases in colony forming units per mL (cfu/mL) occurring as early as 5 min following exposure. A very modest post-antibiotic effect is seen only at high

concentrations against *P. aeruginosa* [25]. Recently, Bulitta *et al.* demonstrated both the rate and extent of killing by colistin are decreased at high compared to low inocula [21]. Using a genetically characterized isolate of *P. aeruginosa* (PAO1), killing of the susceptible population at an inoculum of 10⁹ or 10⁸ cfu/mL was 23-fold and 6-fold slower, respectively, compared with an inoculum of 10⁶ cfu/mL. At the 10⁹ inoculum, up to 32-fold higher concentrations were required to achieve bactericidal activity (≥3-log₁₀ cfu/mL decrease) compared with the 10⁶ inoculum.

Despite the often extensive initial killing observed against colistin-susceptible strains with exposure to colistin alone, regrowth is a common feature both *in vitro* [22,24,26,27] and *in vivo* [28]. Regrowth of *A. baumannii* [23] and *K. pneumoniae* [24] has been reported in static time-kill studies utilizing colistin concentrations up to 64× MIC, while Gunderson *et al.* [22] reported regrowth of two MDR but colistin-susceptible clinical isolates of *P. aeruginosa* with colistin concentrations up to 200 mg/L. Such concentrations are well in excess of those that can be safely achieved clinically (discussed subsequently). Colistin heteroresistance, the phenomenon whereby a strain deemed susceptible based upon standard MIC measurements (e.g. MIC ≤2 mg/L) harbors a subpopulation of colistin-resistant cells (e.g. MIC ≥4 mg/L), has been observed in *A. baumannii* [29], *K. pneumoniae* [24], and *P. aeruginosa* (authors unpublished data), and is probably an important contributor to regrowth and the emergence of colistin resistance. In an important study, Li *et al.* [30] demonstrated that colistin-resistant subpopulations in colistin-heteroresistant strains of *A. baumannii* had remarkably greater susceptibility, compared to their parent strains, to other antibiotics including those that normally are not active against Gram-negative bacteria. The potential presence of colistin-resistant subpopulations before therapy, and the observation of rapid amplification of colistin-resistant subpopulations with colistin monotherapy, suggest caution with the use of colistin monotherapy and highlight the importance of investigating rational colistin combinations [4^{••},30].

Integrated pharmacokinetics/ pharmacodynamics

Recent studies investigating the relationship between the PK and PD of colistin have employed dose fractionation to determine which PK/PD index best correlates with antibacterial activity of colistin [28,31[•],32^{••},33^{••}]. Bergen *et al.* [31[•]] performed an extensive investigation in an *in vitro* PK/PD model against three strains of *P. aeruginosa* (including a colistin-susceptible but MDR strain) with analysis based upon unbound (*f*) indices (i.e. *f*C_{max}/MIC, *f*AUC/MIC, and *f*T_{>MIC}). Overall killing was best correlated with *f*AUC/MIC. In a study utilizing a neutropenic mouse thigh infection model, Keththireddy *et al.* [28] reported once-daily dosing of colistin was most effective

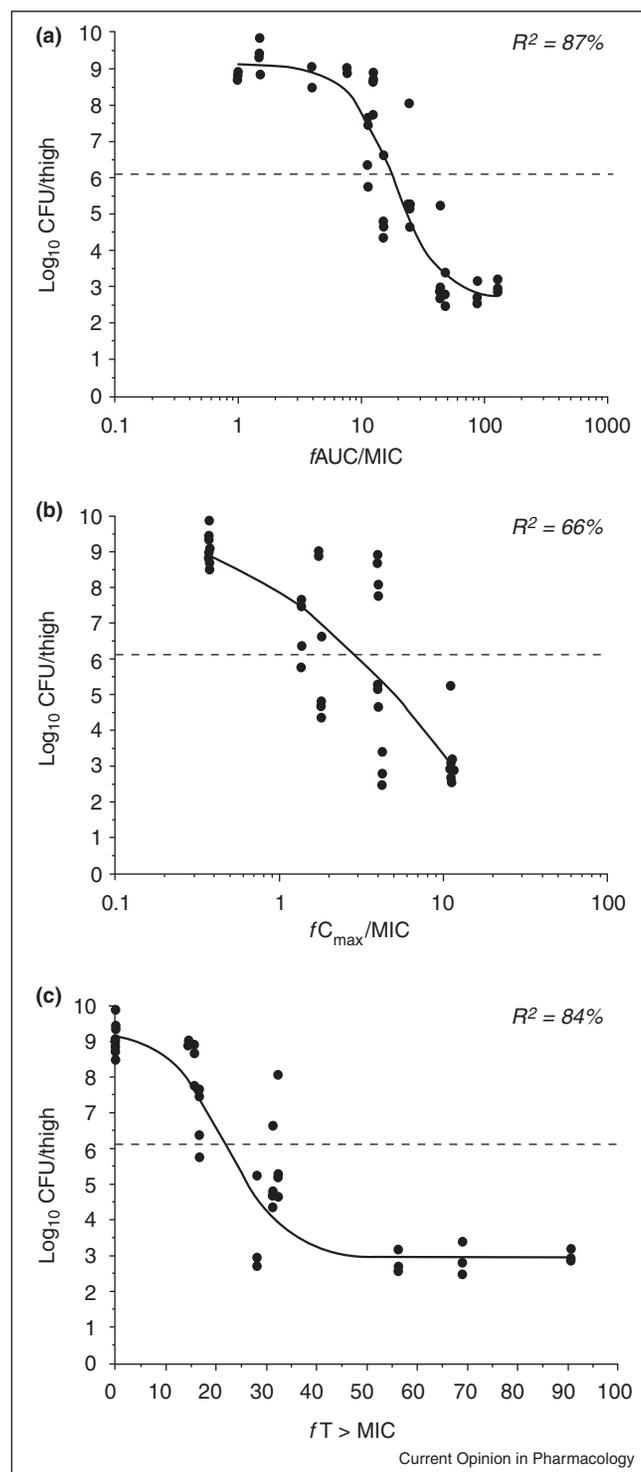
against *P. aeruginosa* suggesting that C_{\max}/MIC may be the PK/PD index most predictive of activity; however that conclusion could not be confirmed since PK data were not available. Dudhani *et al.* [32^{••},33^{••}] employed neutropenic mouse thigh and lung infection models and determined the time course of total (i.e. protein-bound plus unbound) and unbound plasma colistin concentrations. This allowed the PK/PD analysis to be based upon unbound indices. Against three strains of each of *P. aeruginosa* and *A. baumannii* (including MDR but colistin-susceptible and, for *A. baumannii*, colistin-heteroresistant strains), $f\text{AUC}/\text{MIC}$ was the index most predictive of the antibacterial effect in both thigh and lung infection models (Figure 1), in agreement with *in vitro* data [31[•]]. Thus it appears that time-averaged exposure to colistin is important for its antibacterial activity. It is of note that the $f\text{AUC}/\text{MIC}$ values associated with a given magnitude of effect (e.g. 2- \log_{10} reduction in viable bacteria) were generally similar across the *in vitro* and *in vivo* models, the various strains of the two bacterial species and the infection sites [31[•],32^{••},33^{••}]. For example, the colistin $f\text{AUC}/\text{MIC}$ values required to achieve 2- \log_{10} kill against 3 strains of *P. aeruginosa* in both thigh and lung infection models ranged from only 27.6 to 45.9 [32^{••}]. The identification that $f\text{AUC}/\text{MIC}$ is the predictive index and the values of this index for different magnitudes of effect will be key to designing optimal dosage regimens for patients as more information arises on population PK in humans.

Are current dosage regimens optimal?

The simple answer to the abovementioned question is 'no' and much confusion has surrounded the 'optimal' dosing of colistin [4^{••},5]. With its role as a 'salvage' therapy for otherwise untreatable infections, and with resistance to colistin beginning to emerge, it is crucial that CMS is administered in regimens that maximize antibacterial activity and minimize resistance development, while also minimizing the potential for adverse effects (e.g. nephrotoxicity). Fortunately, progress has been made recently in understanding of the PK of CMS and formed colistin in various categories of patients.

It is now evident that the currently used dosage regimens of CMS are likely to generate suboptimal exposure to colistin in many patients across various patient groups. Li *et al.* [15] reported that cystic fibrosis patients administered intravenous CMS had a range of peak plasma concentrations (C_{\max}) of formed colistin of 1.2–3.1 mg/L at steady-state. Importantly, even before consideration of protein binding plasma colistin concentrations in many cases failed to reach the CLSI breakpoint of 2 mg/L [34] defining susceptibility to colistin for *P. aeruginosa* and *A. baumannii*. Dosage regimens of CMS are also suboptimal in many critically ill patients. Markou *et al.* [35] and Imberti *et al.* [36] reported plasma colistin C_{\max} at steady-state of 1.15–5.14 mg/L and 0.68–4.65 mg/L,

Figure 1



Relationships for *P. aeruginosa* ATCC 27853 between the log₁₀ cfu per thigh at 24 h and the PK/PD indices (a) $f\text{AUC}/\text{MIC}$, (b) fC_{\max}/MIC and (c) $fT > \text{MIC}$. Each symbol represents the mean datum per mouse from two thighs. R^2 is the coefficient of determination. The dotted line represents the mean bacterial burden in thighs at the start of treatment. Reproduced from Dudhani *et al.* [32^{••}] with permission.

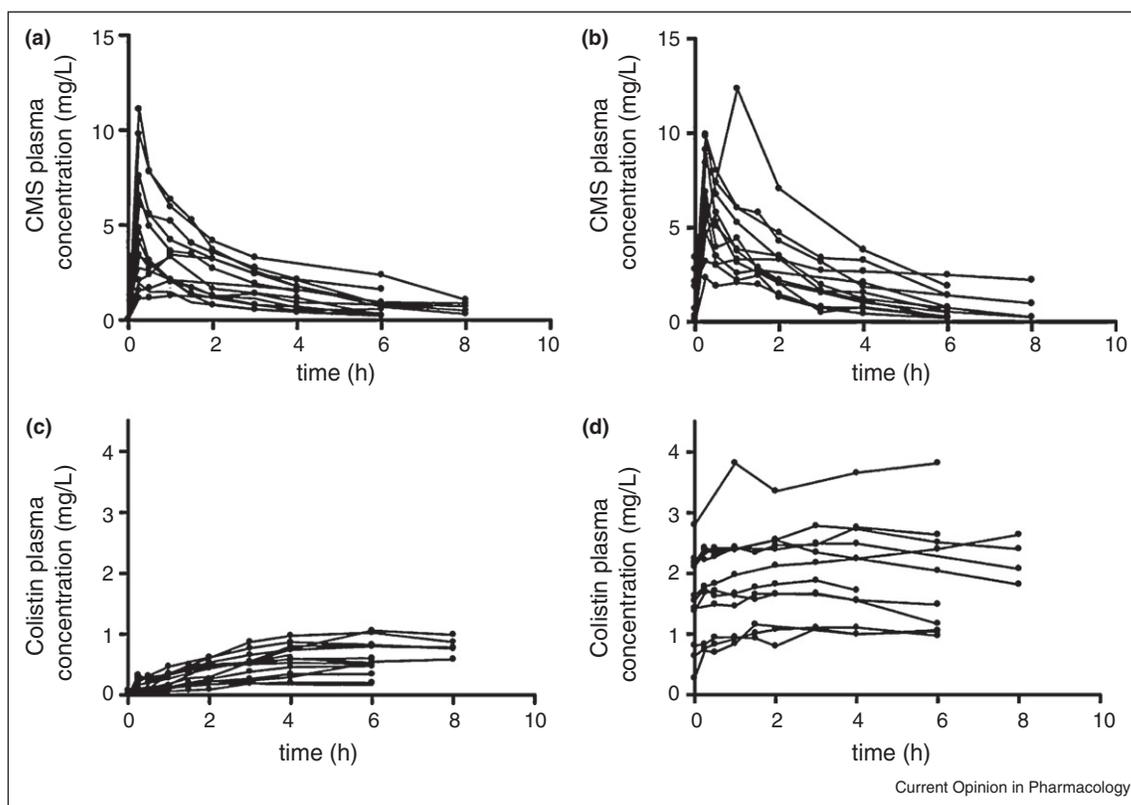
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respectively, in critically ill patients with moderate to good renal function. In 18 renally competent (creatinine clearance >41 mL/min) critically ill patients, Plachouras *et al.* [17**] reported a significant delay in attainment of steady-state plasma concentrations of formed colistin when CMS therapy commenced with maintenance dosing, without administration of a loading dose (Figure 2); plasma colistin concentrations were well below the MIC breakpoints for the first several doses after commencing the maintenance dosage regimen with CMS. Even at steady-state, the typical plasma colistin C_{max} was estimated to be only 2.3 mg/L, with many patients considerably below this concentration. The substantial delay between initiation of CMS therapy and attainment of steady-state observed in that study is significant not only because delayed initiation of appropriate antimicrobial therapy is associated with increased mortality in critically ill patients [37,38], but also because low colistin concentrations have been associated with the amplification of colistin-resistant subpopulations [24,32**,39]. To remedy this situation, the administration of a loading dose of CMS at the commencement of therapy has been suggested [17**,40]. In support of this, mathematical modeling by Bulitta *et al.* [21] predicted colistin regimens with a large

colistin exposure during the first ~ 12 h may be beneficial, providing enough net killing such that the immune system may be able to eradicate any remaining colistin-resistant cells.

In the largest population PK study to date, Garonzik *et al.* [41**,42] have revealed the impact of renal function in 105 critically ill patients on the disposition of CMS and formed colistin. In that study, 89 critically ill patients with very diverse renal function not receiving renal support had average steady-state plasma colistin concentrations of 0.48–10.0 mg/L when receiving CMS daily doses selected by the treating physician (all but three patients received CMS daily doses within the currently recommended range). This study is the first to reveal that creatinine clearance is an important covariate for both the clearance of CMS and the apparent clearance of colistin. As renal function declined in these patients so too did the renal clearance of CMS, resulting in a greater fraction of the administered dose of CMS converted to colistin (hence the apparent clearance of formed colistin was lower in patients with poor renal function). Importantly, administration of CMS at the upper limit of the current product-recommended dose

Figure 2



Observed individual concentrations of CMS (Panels a and b) and formed colistin (Panels c and d) in plasma of critically ill patients after the administration of the first (Panels a and c; fourteen patients) and fourth (Panels b and d; 12 patients) dose of CMS. Reproduced from Plachouras *et al.* [17**] with permission.

range to patients with moderate to good renal function resulted in low and potentially suboptimal plasma colistin concentrations. This is particularly so if the MIC of the infecting organism is in the upper range (i.e. 2 mg/L), or the infection is associated with high bacterial numbers. As suboptimal colistin concentrations may result in amplification of colistin-resistant subpopulations, the most appropriate approach is likely to be therapy with a rationally selected colistin combination regimen [4^{••},30,41^{••}]. In 16 additional patients receiving intermittent hemodialysis (12 patients) and continuous renal replacement therapy (4 patients), both CMS and colistin underwent relatively efficient extracorporeal clearance [41^{••}], which is in agreement with previous case reports [16,43]. A very important practical outcome of this population PK study has been the generation of suggested CMS maintenance doses for various categories of critically ill patients [41^{••}].

Conclusion

As more data accumulate from clinical studies, the integration of PD (clinical cure, bacteriological eradication, development of resistance) and toxicodynamic (e.g. nephrotoxicity) endpoints, together with refinements in population PK models, will help to optimize the administration of this last-line antibiotic in the various categories of patients who require it.

Acknowledgements

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- of special interest
- of outstanding interest

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Colistin Methanesulfonate Is an Inactive Prodrug of Colistin against *Pseudomonas aeruginosa*

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There is a dearth of information on the pharmacodynamics of “colistin,” despite its increasing use as a last line of defense for treatment of infections caused by multidrug-resistant gram-negative organisms. The antimicrobial activities of colistin and colistin methanesulfonate (CMS) were investigated by studying the time-kill kinetics of each against a type culture of *Pseudomonas aeruginosa* in cation-adjusted Mueller-Hinton broth. The appearance of colistin from CMS spiked at 8.0 and 32 mg/liter was measured by high-performance liquid chromatography, which generated colistin concentration-time profiles. These concentration-time profiles were subsequently mimicked in other incubations, independent of CMS, by incrementally spiking colistin. When the cultures were spiked with CMS at either concentration, there was a substantial delay in the onset of the killing effect which was not evident until the concentrations of colistin generated from the hydrolysis of CMS had reached approximately 0.5 to 1 mg/liter (i.e., ~0.5 to 1 times the MIC for colistin). The time course of the killing effect was similar when colistin was added incrementally to achieve the same colistin concentration-time course observed from the hydrolysis of CMS. Given that the killing kinetics of CMS can be accounted for by the appearance of colistin, CMS is an inactive prodrug of colistin with activity against *P. aeruginosa*. This is the first study to demonstrate the formation of colistin in microbiological media containing CMS and to demonstrate that CMS is an inactive prodrug of colistin. These findings have important implications for susceptibility testing involving “colistin,” in particular, for MIC measurement and for microbiological assays and pharmacokinetic and pharmacodynamic studies.

Globally there is a growing threat from the emergence of multidrug-resistant (MDR) microorganisms (7, 15, 18, 19). Although the threat from MDR gram-positive organisms has lessened, at least temporarily, owing to the development of new antimicrobial agents active against these organisms (36), the situation is quite different for MDR gram-negative bacteria (36, 37). With few new antibiotic classes in the drug development pipeline for the treatment of infections caused by MDR gram-negative bacteria, *Pseudomonas aeruginosa* in particular, we are unlikely to see any new advances in the treatment of infections caused by these organisms in the next few years. Unfortunately, MDR *P. aeruginosa* is increasing in prevalence (19, 35, 48); and infections with this organism are causing major clinical problems in patients with burns, neutropenia, or cystic fibrosis and in those who are immunocompromised (14, 47, 48). Several institutions have already experienced outbreaks of *P. aeruginosa* or *Acinetobacter baumannii* infections resistant to all commercially available antibiotics except the polymyxins (3, 5, 39, 46). It is precisely this scenario to which the Infectious Disease Society of America refers in its “Bad Bugs, No Drugs” campaign (18). Given these circumstances, a review of the activities and clinical use of many older antimicrobial drugs is occurring. With their rapid bactericidal activ-

ities and current low levels of resistance (6, 12, 16, 34), the polymyxins, and colistin (also known as polymyxin E) in particular, have undergone a revival as agents for treatment of infections caused by MDR *P. aeruginosa*, *A. baumannii*, and *Klebsiella pneumoniae* (6, 20, 30). Previously relegated to the status of a reserve agent after early reports of a “high” incidence of toxicity (21, 44), colistin is now a last line of defense for the treatment of infections with MDR gram-negative organisms (3, 30, 33).

Colistin is a cationic, multicomponent lipopeptide consisting of a cyclic heptapeptide with a tripeptide side chain acylated at the N terminus by a fatty acid (Fig. 1a). The two major components are colistin A (polymyxin E₁) and colistin B (polymyxin E₂) (41). Two different forms of colistin are available commercially: colistin sulfate (hereafter referred to as colistin) and sodium colistin methanesulfonate (CMS) (Fig. 1b). CMS is produced by the reaction of colistin with formaldehyde and sodium bisulfite (1), which leads to the addition of a sulfomethyl group to the primary amines of colistin. Colistin is primarily used topically, whereas CMS is used parenterally; both forms may be given by inhalation (30). CMS is less toxic than colistin when it is administered parenterally (4, 45), and indeed, this was the reason for the development of CMS. In aqueous solutions CMS undergoes hydrolysis to form a complex mixture of partially sulfomethylated derivatives, as well as colistin (1, 4, 25). By the use of high-performance liquid chromatographic (HPLC) methods for separate quantification of CMS and colistin in biological fluids (26, 27, 29), the formation of colistin in vivo has been demonstrated in patients (24, 31) and rats (28) receiving parenteral CMS; both the CMS administered and the colistin generated circulate in plasma. Further-

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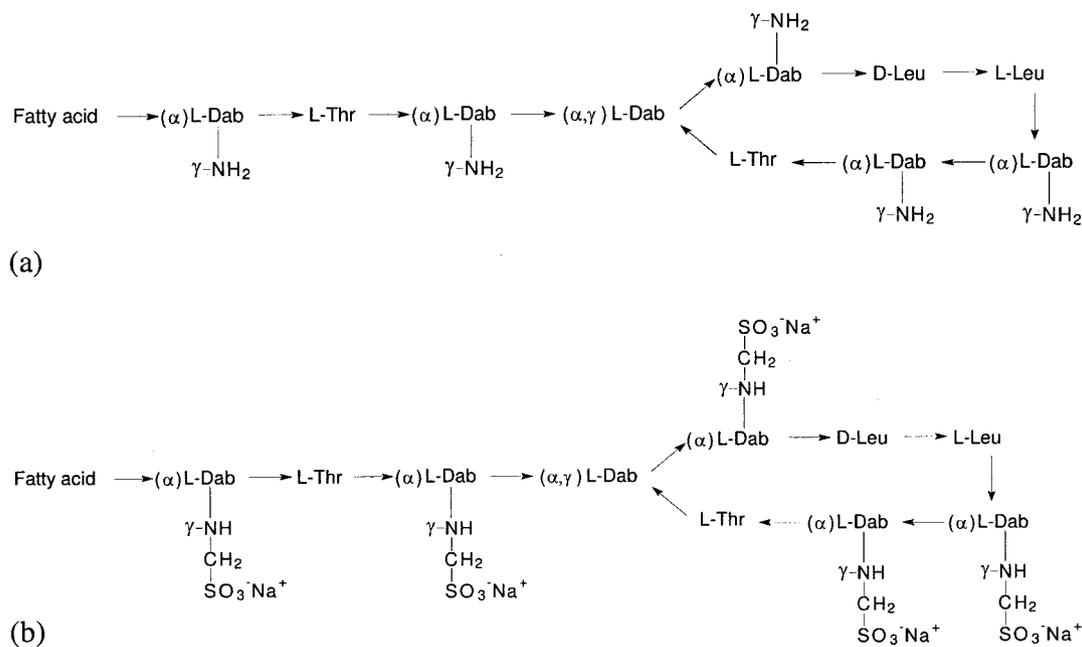


FIG. 1. (a) Structures of colistin A and B; (b) structures of sodium colistin A and B methanesulfonate (CMS). Fatty acid, 6-methyloctanoic acid for colistin A and 6-methylheptanoic acid for colistin B; Thr, threonine; Leu, leucine; Dab, α,γ -diaminobutyric acid. α and γ indicate the respective $-\text{NH}_2$ involved in the peptide linkage.

more, the pharmacokinetics of CMS and colistin have been demonstrated to differ (24, 28, 31).

Even after adjustment for molecular weight differences, CMS has reduced antibacterial activity compared with that of colistin, as assessed by MICs (2, 4, 11, 32, 45), and has been reported to be two to four times less active against *P. aeruginosa* (11, 32, 45). However, to our knowledge, no previous publications have reported on the contribution to bacterial killing made by each of CMS and colistin, the latter being formed via hydrolysis from CMS. Knowledge of the relative activities of CMS and colistin has important implications for standardization of susceptibility studies (e.g., MIC measurement), as well as microbiological assays of "colistin" in biological fluids; pharmacokinetic and pharmacodynamic studies involving "colistin" would also be affected. The aim of this study was therefore to determine the relative contributions of colistin and CMS to activities against *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains. A reference strain of *P. aeruginosa* ATCC 27853 (American Type Culture Collection, Manassas, VA) was used in this study. The strain was stored at -80°C in a cryovial storage container (Simport Plastics, Quebec, Canada). Fresh isolates were subcultured on horse blood agar (Media Preparation Unit, The University of Melbourne, Parkville, Australia) and incubated at 35°C for 24 h prior to each experiment. Calcium-adjusted Mueller-Hinton broth (CAMHB; lot 332998; Oxoid, Hampshire, England) was used.

Chemicals and reagents. Colistin sulfate (lot 072K1656; 19,530 units/mg) was purchased from Sigma-Aldrich (St. Louis, MO); 200-mg/liter and 500-mg/liter stock solutions were prepared in water and stored at 4°C before use. Colistin is stable under these conditions (25). Sodium colistin methanesulfonate (lot A1680552; 13,100 units/mg) was purchased from Alpharma Pharmaceuticals (Copenhagen, Denmark); stock solutions of 1,000 mg/liter in water were freshly prepared before each experiment to minimize the potential hydrolysis of CMS in aqueous solutions (25). All stock solutions were filtered by using 0.22- μm -pore-

size Millex-GP filters (Millipore, Bedford, MA). All other chemicals were from the suppliers described previously (26).

Time-kill kinetics. The MIC of colistin (sulfate) and the apparent MIC of CMS for this strain, as determined by broth microdilution (8), were 1 and 4 mg/liter, respectively.

(i) **Colistin methanesulfonate.** Time-kill studies were conducted with two concentrations of CMS (8.0 and 32 mg/liter) that corresponded to two and eight times the apparent MIC of CMS against *P. aeruginosa* ATCC 27853, respectively. An aliquot (200 μl) of an overnight culture was added to 20 ml of CAMHB and incubated at 37°C until early log-phase growth was reached; 5 ml was then transferred to a 500-ml bottle (Schott Duran, Germany) containing 385 ml of CAMHB, which gave approximately 5×10^5 CFU/ml. CMS was added to achieve an initial concentration of either 8.0 or 32 mg/liter. The experiment was conducted for 240 min in a shaking water bath (100 rpm) at 37°C . Serial samples were obtained at 0, 30, 60, 75, 90, 120, 135, 150, 165, 180, 210, and 240 min (2 ml per sample) for viable cell counting and determination of colistin concentrations and, where relevant, CMS concentrations (see below). Viable cell counting was conducted by spiral plating (WASP2 spiral plater; Don Whitley Scientific Ltd., England) 50 μl of appropriately diluted sample onto nutrient agar plates (Media Preparation Unit), followed by incubation at 35°C for 18 to 24 h. The colonies were counted with a ProtoCOL colony counter (Don Whitley Scientific Ltd.); the limit of detection was 20 CFU/ml. For each CMS concentration, three replicates were performed, each on a separate day. The time course of the concentrations of colistin formed by hydrolysis from CMS at each concentration were used as the basis for time-kill experiments involving colistin, as described below.

(ii) **Colistin.** The media and inoculum were prepared as described above for CMS. After the inoculation, colistin (sulfate) solution was added at 5-min intervals to mimic the concentration-time course of colistin produced by hydrolysis of CMS at 8.0 or 32 mg/liter, determined as described above (corrections were made to allow for differences in molecular weights between the base and the sulfate forms of colistin). Aliquots from either the 200-mg/liter or 500-mg/liter colistin solutions were used. The experiment was conducted for 240 min in a shaking water bath (100 rpm) at 37°C . Serial samples were obtained at 0, 30, 60, 75, 90, 105, 120, 135, 150, 165, 180, 210, and 240 min (2 ml per sample) for viable cell counting and determination of colistin concentrations. Three replicates were performed at each concentration, each on a separate day. In a control experiment, colistin was added to achieve an initial concentration of 6.0 mg/liter (six times the MIC of colistin). Serial samples were obtained at 0, 5, 10, 15, 20, 30, 45, 60, 90, and 120 min for viable cell counting (see above).

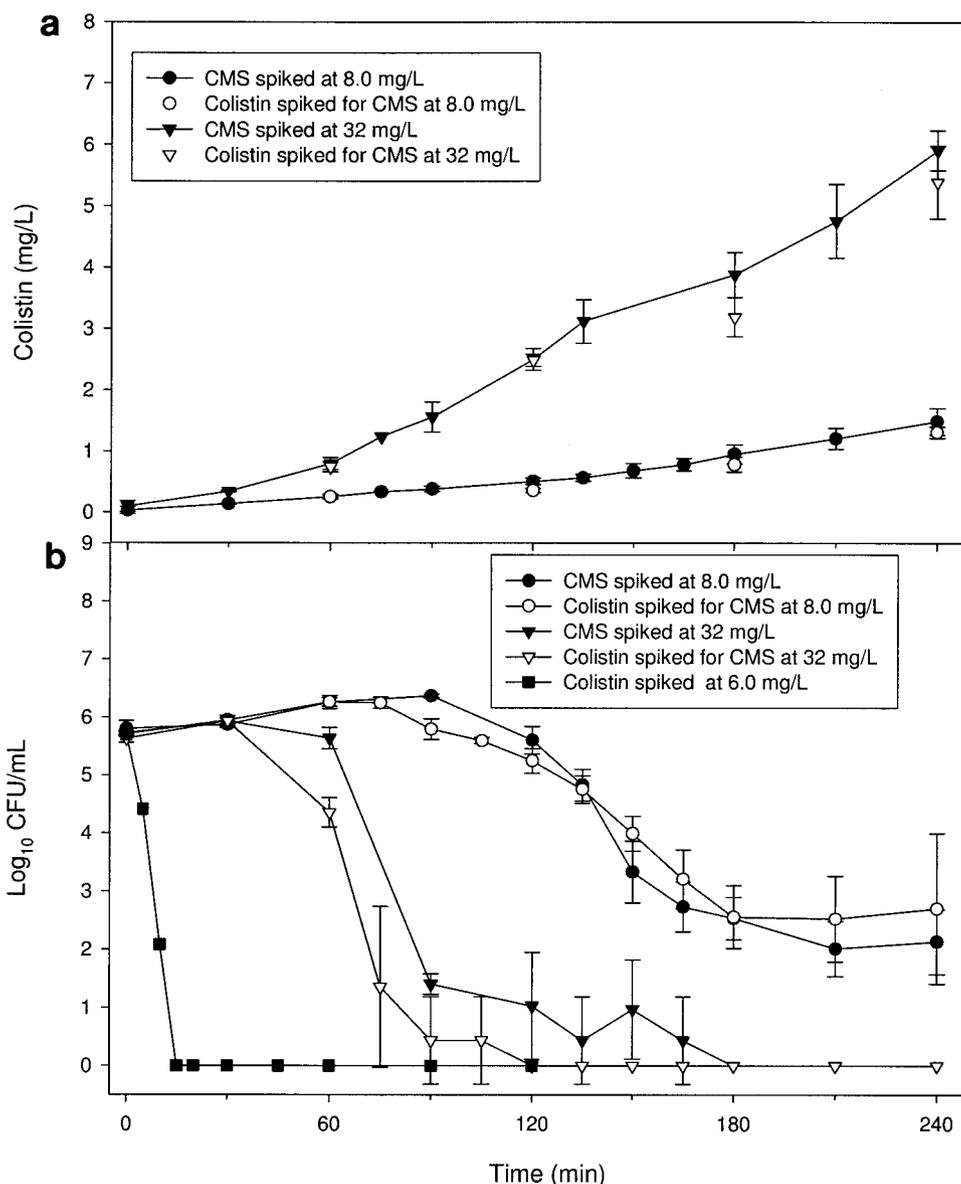


FIG. 2. (a) Concentration-time course of colistin produced from CMS spiked at 8.0 mg/liter and 32 mg/liter ($n = 3$) and from incremental spiking with colistin ($n = 3$); (b) time-kill curves for *P. aeruginosa* obtained by using colistin methanesulfonate spiked at 8.0 mg/liter and 32 mg/liter ($n = 3$) at zero time, spiked incrementally with colistin to mimic the colistin concentration-time course achieved after spiking of the sample with colistin methanesulfonate at 8.0 mg/liter and 32 mg/liter ($n = 3$), and colistin spiked at 6.0 mg/liter at zero time ($n = 1$; control experiment).

Determination of colistin and CMS in CAMHB. Samples from the time-kill studies were taken in duplicate (250 μ l for colistin and 150 μ l for CMS) and placed in 1.5-ml microcentrifuge tubes (Neptune; CLP, Mexico) and immediately stored at -80°C . The concentrations of colistin and CMS were measured by two sensitive HPLC methods previously developed by our group, with minor modifications (26, 27, 29). The assay ranges were 0.083 mg/liter to 5.80 mg/liter for colistin (base) and 0.25 mg/liter to 40 mg/liter for CMS. The accuracy and reproducibility of the results for the quality control samples for both assays fell within 10% of the target values.

Data analysis. The killing effects from addition of CMS or colistin were examined descriptively and were quantified by calculation of the area under the concentration-time curve of log_{10} CFU/ml from 0 to 240 min (AUC_{0-240}) normalized by log_{10} CFU/ml _{$t=0$} , where log_{10} CFU/ml _{$t=0$} is the initial log_{10} CFU/ml value; this was not performed for the control experiment, where colistin was added at 6.0 mg/liter at zero time and samples were collected for 120 min. AUC_{0-240} was calculated by using the linear trapezoidal rule.

RESULTS

Time course of colistin formation from CMS. The initial CMS concentrations achieved were 7.64 ± 0.64 mg/liter ($n = 3$) for 8.0 mg/liter (two times the apparent MIC of CMS) and 29.0 ± 1.98 mg/liter ($n = 3$) for 32 mg/liter (eight times the apparent MIC of CMS). Figure 2a shows the mean colistin concentrations present after the samples were spiked with CMS. The concentrations of colistin present immediately following addition of CMS at 8.0 mg/liter were below the limit of quantification of 0.083 mg/liter for two of three samples and 0.10 mg/liter for the remaining sample; the concentrations present immediately following addition of CMS at 32 mg/liter

TABLE 1. AUC_{0-240} of killing curves normalized by baseline \log_{10} CFU/ml_{t = 0}

Colistin form	$AUC_{0-240}/(\log_{10} \text{CFU/ml}_t = 0)$ ($n = 3$)	
	8.0 mg/liter	32 mg/liter
CMS	186.3 ± 6.0	90.4 ± 4.1
Colistin ^a	192.8 ± 10.4	70.0 ± 7.4

^a Spiked incrementally to achieve the same concentration-time course of colistin observed from hydrolysis of CMS spiked initially at 8.0 mg/liter or 32 mg/liter.

were 0.17 and 0.12 mg/liter for two of three samples and below the limit of quantification for the remaining sample. This indicates that the amount of colistin present in the batch of CMS used was very low. Following addition of CMS, comparatively small amounts of colistin were formed in the first 60 min of incubation: 0.26 ± 0.04 mg/liter from 8.0-mg/liter CMS and 0.80 ± 0.10 mg/liter from 32-mg/liter CMS. After 240 min of incubation, these values had risen to 1.49 ± 0.22 mg/liter (~1.5 times the MIC of colistin) from 8.0-mg/liter CMS and 5.91 ± 0.32 mg/liter (~6 times the MIC of colistin) from 32-mg/liter CMS; this corresponds (in molar terms) to $29.1\% \pm 2.1\%$ and $30.5\% \pm 2.2\%$, respectively, of the CMS being converted to colistin. The concentration-time profiles of colistin achieved by using incremental spiking of colistin matched closely those derived from the hydrolysis of CMS in CAMHB (Fig. 2a).

Time-kill kinetics. The time-kill profiles achieved with CMS and colistin at each concentration are shown in Fig. 2b. In the control experiment in which colistin was spiked at 6.0 mg/liter (six times the MIC of colistin), no viable bacteria were detectable after 15 min. When CMS was spiked at 8.0 mg/liter, killing began at approximately 90 min when the concentration of colistin formed by hydrolysis of CMS was approximately 0.5 the MIC of colistin (0.46 ± 0.05 mg/liter of colistin). When CMS was spiked at 32 mg/liter, killing began at approximately 30 min, with a rapid decline in CFU/ml at 60 min, when the concentration of colistin formed by hydrolysis of CMS was approximately 1.0 the MIC of colistin (0.96 ± 0.12 mg/liter of colistin). In both cases, very similar time-kill profiles occurred when colistin was added incrementally to achieve the colistin concentration-time courses observed from the hydrolysis of CMS spiked initially with either 8.0 or 32 mg/liter CMS. The mean AUC_{0-240} values normalized by initial \log_{10} CFU/ml, $AUC_{0-240}/(\log_{10} \text{CFU/ml}_t = 0)$, are shown in Table 1.

DISCUSSION

“Colistin” is now a last line of defense against infections caused by MDR gram-negative organisms (3, 30, 33, 35). Despite renewed interest in the clinical use of “colistin”, confusion has surrounded its use in susceptibility studies, in particular MIC measurement, as well as in microbiological assays used to measure “colistin” concentrations in biological fluids. Information on the pharmacokinetics and pharmacodynamics of CMS and colistin, especially in critically ill patients, is also lacking (31, 38). Study of the antibacterial activity of CMS, the parenteral form of colistin, has proven complicated due to the hydrolytic conversion of CMS to colistin (25); and this is reflected in the literature (30). Prior to the present study, the

relative contributions of CMS and colistin to antibacterial activity have, to our knowledge, never been directly investigated.

The concentrations of CMS chosen for the present study (8.0 and 32 mg/liter) represent the concentrations achievable in plasma in vivo (24, 31). A control experiment in which colistin was spiked at 6.0 mg/liter (six times the MIC of colistin), equivalent to the concentration of colistin generated in 240 min after spiking of the samples with CMS at 32 mg/liter (Fig. 2a), demonstrated rapid and extensive killing such that the number of CFU/ml had fallen below the limit of detection of counting of viable organisms by 15 min (Fig. 2b); this is consistent with the findings of our previous report (32). In sharp contrast, when CMS was spiked at zero time to achieve concentrations equivalent to two and eight times the apparent MIC for CMS, there was a substantial delay in the onset of the killing effect (Fig. 2b); in both cases, growth continued for some time after addition of CMS (~75 min for CMS spiked at 8.0 mg/liter and ~30 min for CMS spiked at 32 mg/liter). This is significant, given that a concentration of CMS even eight times its apparent MIC was unable to cause bacterial killing until significant amounts of colistin had formed. As has already been shown with concentration-dependent anti-infectives, including colistin, bacterial killing occurs at concentrations below the MIC (32, 40). At both CMS concentrations, killing was not evident until the concentrations of colistin generated from the hydrolysis of CMS had reached approximately 0.5 to 1 mg/liter (i.e., ~0.5 to 1 times the MIC for colistin).

Importantly, the time course of the killing effect achieved by spiking with CMS at zero time (8.0 mg/liter or 32 mg/liter) was very similar to that observed when colistin was added incrementally to achieve the same colistin concentration-time course from the hydrolysis of CMS (Fig. 2). In particular, the early parts of the killing curves generated from CMS are virtually superimposable on the respective curves from incremental spiking of colistin; this is a time when very little colistin has been generated from CMS. Given that killing did not begin until significant amounts of colistin had formed, as mentioned above, it appears that CMS and the partially sulfomethylated derivatives possess little, if any, antibacterial activity. For each concentration of CMS (8.0 and 32 mg/liter), the $AUC_{0-240}/(\log_{10} \text{CFU/ml}_t = 0)$ obtained by spiking of the samples with CMS or spiking of the samples incrementally with colistin to achieve the same colistin concentration-time course (Table 1) were within 3.5% and 22.5%, respectively. Consequently, the time course of antibacterial activity from CMS can be accounted for by the appearance of colistin. Thus, our study has clearly demonstrated that at both concentrations of CMS (8.0 and 32 mg/liter), antipseudomonal activity was due to the formation of colistin; CMS alone displayed no antibacterial activity. CMS may therefore be regarded as an inactive prodrug of colistin.

The present study has demonstrated that the formation of colistin in vivo following administration of CMS is a prerequisite for antibacterial activity. It is also clear that the in vitro hydrolysis of CMS to colistin during microbiological procedures conducted in the laboratory (e.g., MIC measurement) has the potential to make CMS appear to possess antibacterial activity. Although we recently reported for the first time colistin formation from CMS in vitro (25) and in vivo (24, 28, 31), to our knowledge no previous work has demonstrated the

formation of colistin in microbiological media spiked with CMS during incubation. In the present study, approximately 30% of the CMS present was hydrolyzed to colistin after only 240 min. In microbiological procedures such as MIC measurement, where similar temperature conditions but significantly longer incubation periods are used (up to 24 h for MIC measurement), even more colistin would be expected to form. This, together with our demonstration that CMS possesses no (or little) antibacterial activity, has very important implications for microbiological testing procedures.

Due to uncertainties over whether CMS possesses antibacterial activity in its own right, MIC measurements for "colistin" have been performed by using colistin (8) or CMS (6), or both (11, 32). This has caused confusion for clinicians and clinical microbiologists, as evidenced by discussions on the American Society for Microbiology e-mail discussion group (ClinMicroNet, 17 May 2005), with questions such as the relevance of an MIC test performed with colistin for a patient receiving CMS remaining unanswered. The present study has demonstrated that the use of CMS is inappropriate for MIC measurement, as antimicrobial activity is due to the formation of colistin and not to the CMS itself. Recently the Clinical and Laboratory Standards Institute published MIC measurement protocols and stated that colistin, not CMS, should be used when MICs are determined (8). This decision appears to be justified by our results. Given the emergence of resistance to colistin (32) and its increasing use, accurate susceptibility data will be vital for the meaningful inclusion of "colistin" in the testing lists of antimicrobial susceptibility studies; currently, "colistin" is seldom included in such studies (43).

The demonstration that colistin forms in microbiological media spiked with CMS also has important implications for measurement of "colistin" concentrations in biological fluids by microbiological assays. Current recommendations for the microbiological assay of "colistin" involve diffusion methods that use long periods of incubation at elevated temperatures (up to 24 h at 37°C) (23, 50), and significant amounts of colistin are likely to form via hydrolysis from CMS under these conditions. After parenteral administration of CMS, *in vivo* hydrolysis ensures that both CMS and colistin will be present at the time of collection of a blood sample (24, 28, 31). Over the duration of a microbiological assay, however, significant amounts of colistin will continue to form. Regardless of whether CMS or colistin is chosen as the reference standard against which biological samples from patients are compared, such assays are unable to differentiate between the colistin present at the time of sampling and the colistin formed via the hydrolysis of CMS during incubation. Consequently, microbiological assays of samples collected from patients administered CMS give no information about the individual concentrations of colistin and CMS present at the time of collection.

The inability to accurately determine colistin and CMS concentrations in biological fluids by analytical methods such as microbiological assay, as described above, has significant implications for past and future research on the pharmacokinetics and pharmacodynamics of "colistin." Many of the data on the pharmacokinetics of "colistin" that have been generated were obtained by microbiological assays (9, 13, 52). Some pharmacokinetic studies used HPLC methods that are potentially more specific for measurement of "colistin" concentrations in

humans (22, 42); however, these particular HPLC methods suffer from problems similar to those encountered when microbiological assays are used. In particular, such HPLC methods (42) cannot differentiate between the colistin present at the time of sampling and the colistin formed by hydrolysis subsequent to sample collection. Thus, most pharmacokinetic data on "colistin" published to date can be considered representative of a complex mixture of colistin and CMS and its partially sulfomethylated derivatives. Given that colistin formation from CMS occurs *in vivo* (24, 31) and, as demonstrated in the present study, that CMS is an inactive prodrug, future studies attempting to define pharmacokinetic and pharmacodynamic parameters must rely on assays that are capable of distinguishing between colistin and CMS, such as the HPLC methods described previously (26, 27).

An explanation as to why CMS displays little, if any, antimicrobial activity may reside in the postulated mechanism of action of the cationic peptides, which includes the polymyxins (16). For gram-negative microorganisms, it is proposed that the antibacterial activity of polymyxins involves a two-step process that begins with the displacement of divalent cations (Ca^{2+} , Mg^{2+}) on cell surface lipopolysaccharides of the outer membrane, followed by interaction with the negatively charged cytoplasmic membrane (16); the increasing net positive charge of the peptide promotes interaction with each membrane (17, 51). Given that at physiological pH the net charge on CMS is -5 , the charges on its partially sulfomethylated derivatives range from -3 to $+3$ (-3 with four attached sulfomethyl groups, $+3$ with one attached sulfomethyl group), whereas the net charge on colistin is $+5$, the strongly cationic colistin would be expected to have greater antibacterial activity. While we acknowledge both the limitations of the present study to draw definitive conclusions on this issue and the fact that structural parameters other than charge are important for antibacterial activity in most peptides (10, 17, 49, 51), the postulated mechanism of action of the cationic peptides combined with the polycationic nature of colistin may explain why CMS acts only as a prodrug.

In conclusion, we have demonstrated the formation of colistin during incubation in microbiological media spiked with CMS by sensitive and specific HPLC methods for the quantification of colistin and CMS. Using a reference strain of *P. aeruginosa*, we investigated the contribution to antimicrobial activity of CMS and its hydrolysis product, colistin, which forms in solution from CMS. Our conclusion that CMS is an inactive prodrug of colistin, as well as our demonstration that colistin forms from CMS in microbiological media, has important implications for susceptibility testing involving "colistin," in particular, for MIC measurement, as well as for microbiological assays and pharmacokinetic and pharmacodynamic studies.

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Comparison of once-, twice- and thrice-daily dosing of colistin on antibacterial effect and emergence of resistance: studies with *Pseudomonas aeruginosa* in an *in vitro* pharmacodynamic model

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Objectives: The optimal dosing regimen for colistin methanesulphonate (CMS) against *Pseudomonas aeruginosa* is unknown. CMS is converted *in vivo* to its active form, colistin. We evaluated three colistin dosage regimens in an *in vitro* pharmacokinetic/pharmacodynamic model.

Methods: Three intermittent dosage regimens involving 8, 12 and 24 h dosage intervals (C_{\max} of 3.0, 4.5 or 9.0 mg/L, respectively) were employed. Antibacterial activity and emergence of resistance were investigated over 72 h using two strains of *P. aeruginosa*: ATCC 27853 and 19056. The areas under the killing curves (AUBC₀₋₇₂) and population analysis profiles (AUCPAP) were used to compare regimens.

Results: No difference in bacterial killing was observed among different regimens. For ATCC 27853, substantial killing was observed after the first dose with less killing after subsequent doses irrespective of regimen; regrowth to between 5.95 and 7.49 log₁₀ cfu/mL occurred by 72 h (growth control 7.46 log₁₀ cfu/mL). AUCPAPs at 72 h for the 12 hourly (4.08 ± 1.54) and 24 hourly (4.16 ± 2.48) regimens were substantially higher than that for both the growth control (1.63 ± 0.08) and 8 hourly regimen (2.30 ± 0.87). For 19056, bacterial numbers at 72 h with each regimen (1.32–2.75 log₁₀ cfu/mL) were far below that of the growth control (7.79 log₁₀ cfu/mL); AUCPAPs could not be measured effectively due to the substantial killing.

Conclusions: No difference in overall bacterial kill was observed when the recommended maximum daily dose was administered at 8, 12 or 24 h intervals. However, the 8 hourly regimen appeared most effective at minimizing emergence of resistance.

Keywords: colistin methanesulphonate, dosage regimens, pharmacokinetics, pharmacodynamics, multidrug resistance

Introduction

The world is facing a growing threat from multidrug-resistant (MDR) microorganisms, especially Gram-negative bacteria,¹⁻³ and several institutions have already experienced outbreaks of MDR Gram-negative bacteria resistant to all commercially available antibiotics except the polymyxins.⁴⁻⁸ The result has been the increasing use of colistin (also known as polymyxin E) as an agent of last resort for treating infections caused by MDR Gram-negative organisms.^{4,9-11} However, knowledge of the pharmacokinetics (PK) and pharmacodynamics (PD) of colistin

is limited, and resistance to the polymyxins has recently emerged.¹²⁻¹⁶ With few new therapeutic options becoming available in the foreseeable future, particularly for *Pseudomonas aeruginosa*,² solid PK/PD data on colistin are urgently needed. Such information will be crucial in determining optimal dosing strategies to maximize the clinical benefit of, minimize the development of resistance to, and prolong the usefulness of this increasingly important therapeutic option.

Colistin is available commercially as colistin sulphate (hereafter referred to as colistin) and sodium colistin methanesulphonate (CMS). Owing to reduced toxicity when compared with

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colistin,^{17,18} CMS is used parenterally whereas colistin is primarily used topically. The formation of colistin *in vivo* following parenteral administration of CMS has been demonstrated in both rats¹⁹ and humans.^{20,21} Recently, we established that CMS is an inactive prodrug of colistin.²²

Due to the limited knowledge of the PK and PD of colistin and CMS, confusion surrounds the optimal dosing regimen that maximizes antibacterial activity and minimizes the emergence of resistance.^{8,10} At present, 8,^{7,23–25} 12²⁴ and 24 hourly^{26,27} dosage regimens of CMS are all used clinically in patients with normal renal function. The aim of this study was to evaluate the PD of colistin against *P. aeruginosa* in terms of antibacterial activity and emergence of resistance. This was achieved by simulating, in an *in vitro* PK/PD model, the PK of colistin formation in humans administered three clinically relevant dosage regimens of CMS, including the currently recommended regimens.^{28,29} Given that the antibacterial activity of CMS results from its hydrolysis to colistin,²² the PK/PD parameters used to describe the activity of ‘colistin’ must be based on the concentrations of colistin present, not CMS. The PK parameters (C_{\max} and $t_{1/2}$) used in our studies were based on reliable clinical PK data for colistin.³⁰ This study was not designed to determine the optimal PK/PD index for colistin.

Materials and methods

Bacterial strains and media

Two strains of *P. aeruginosa* were employed in this study: a reference strain, ATCC 27853 (American Type Culture Collection, Rockville, MD, USA) and a clinical isolate, 19056 (mucoid) from a patient with cystic fibrosis. The MICs of colistin (sulphate), as determined by broth microdilution,³¹ were 1 mg/L for ATCC 27853 and 0.5 mg/L for 19056; both strains were stored in tryptone soy broth (Oxoid Australia, West Heidelberg, Victoria, Australia) with 20% glycerol (Ajax Finechem, Seven Hills, NSW, Australia) at -80°C in cryovial storage containers (Simport Plastics, Boleil, Quebec, Canada). Prior to each experiment, strains were subcultured onto horse blood agar (Media Preparation Unit, The University of Melbourne, Parkville, Australia) and incubated at 35°C for 24 h. One colony was then selected and grown overnight in 10 mL of cation-adjusted Mueller–Hinton broth (CAMHB; Oxoid, Hampshire, England) from which early log-phase growth was obtained.

Chemicals and reagents

Colistin sulphate was purchased from Sigma-Aldrich (Lot: 095K1048, St Louis, MO, USA; 20 195 U/mg). Immediately prior to each experiment, colistin stock solutions were prepared using Milli-Q water (Millipore Australia, North Ryde, NSW, Australia), sterilized by a 0.22 μm Millex-GP filter (Millipore, Bedford, MA, USA), and then stored at 4°C before use; colistin is stable under these conditions.³² All other chemicals were from suppliers previously described.³³

In vitro PK/PD model

The studies examined the effect of three different colistin dosing regimens (discussed subsequently) on microbiological response and emergence of resistance and were conducted over 72 h using a one-compartment *in vitro* PK/PD model. Briefly, the

system consisted of four sealed reservoirs (compartments) each containing 100 mL of CAMHB (Ca^{2+} 23.0 mg/L and Mg^{2+} 12.2 mg/L) and a magnetic stir bar to ensure adequate mixing. Each experiment was conducted using three replicates, with the remaining (drug-free) reservoir acting as a control to define growth dynamics in the absence of colistin. All reservoirs were heated in paraffin oil to 37°C throughout the experiment. A peristaltic pump (Masterflex® L/S®, Cole-Parmer, USA) was used to deliver sterile (drug-free) CAMHB from a separate sealed reservoir into each of the four compartments at a predetermined rate (0.3 mL/min), displacing an equal volume of CAMHB into a waste receptacle. This produced a $t_{1/2}$ of 4 h for colistin administered into the central reservoirs; this approximates the $t_{1/2}$ determined in cystic fibrosis patients with normal renal function.²⁰ At the beginning of each experiment, a 1.0 mL aliquot of early log-phase bacterial suspension, obtained from overnight culture, was inoculated into each reservoir giving $\sim 10^6$ cfu/mL. Colistin was administered to each treatment reservoir to achieve the desired C_{\max} as described below and in Table 1. Serial samples (1 mL) were collected aseptically from each reservoir via a rubber septum-sealed port for viable cell counting and population analysis profiles (PAP), as well as determination of colistin concentrations (discussed subsequently).

Three intermittent colistin dosage regimens were simulated (Table 1). At the beginning of each experiment, the appropriate loading dose of colistin (sulphate) was injected into three of the four reservoirs followed by intermittent maintenance doses at 8, 12 or 24 h intervals. The 8 hourly dosage regimen closely simulated the expected plasma unbound peak ($C_{\max} = 3$ mg/L)²⁰ and trough ($C_{\min} = 0.75$ mg/L) concentrations of colistin at steady state when CMS is administered 8 hourly according to the manufacturer’s recommendations [5 mg/kg/day of colistin base activity; Coly-Mycin™ M Parenteral package insert (Monarch Pharmaceuticals, Bristol, TN, USA)] in patients with normal renal function. The 12 and 24 hourly dosage regimens were designed to achieve higher C_{\max} values (Table 1) with extended dosage intervals.

Microbiological response and the emergence of resistance to colistin

Sampling times are shown in Table 1. Viable counting and PAP 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 in *in vitro* PK/PD model) or Mueller–Hinton agar (PAP), followed by incubation at 35°C for 24 h. PAP 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.²⁰ Enumeration was performed using a ProtoCOL colony counter (Don Whitley Scientific Ltd); the limit of detection was 20 cfu/mL.

Determination of colistin concentration in CAMHB

Samples (250 μL) collected from the *in vitro* PK/PD experiments were placed in 1.5 mL microcentrifuge tubes (Neptune™, CLP, Mexico) and immediately stored at -80°C until analysis. Concentrations of colistin were measured using HPLC.^{33,34} The assay range for colistin was 0.10–6.00 mg/L; samples were diluted when the expected colistin concentrations were higher than the upper limit of quantification. Analysis of quality control samples with

PK/PD of colistin against *Pseudomonas aeruginosa*

Table 1. Colistin (sulphate) dosage regimens, PK/PD indices and sampling times in the *in vitro* PK/PD model

	8 hourly dosing	12 hourly dosing	24 hourly dosing
Loading dose (mg)	0.30	0.45	0.90
Maintenance dose (mg)	0.23	0.39	0.89
Target C_{max}/C_{min} (mg/L)	3.0/0.75	4.5/0.56	9.0/0.14
ATCC 27853/clinical isolate 19056			
AUC/MIC ^a	39.0/77.9	45.4/91.0	51.1/102.3
C_{max}/MIC^a	3.0/6.0	4.5/9.0	9.0/18.0
$t > MIC^a$	79.3/100	72.3/100	52.8/69.5
Sampling times (h) for microbiological measurements ^b	0, 1, 2, 4, 6, 8, 9, 16, 17, 24, 25, 26, 28, 30, 32, 33, 40, 41, 48, 49, 50, 52, 54, 56, 57, 64, 65 and 72	0, 1, 2, 4, 6, 8, 12, 13, 24, 25, 26, 28, 30, 32, 36, 37, 48, 49, 50, 52, 54, 56, 60, 61 and 72	0, 1, 2, 4, 6, 8, 24, 25, 26, 28, 30, 32, 48, 49, 50, 52, 54, 56 and 72

^aTarget values of PK/PD indices.

^bcfu/mL determined for all samples. PAP were performed at times 0, 24, 48 and 72 h only.

nominal concentrations of 0.40 and 4.00 mg/L had measured concentrations of 0.34 ± 0.03 mg/L ($n = 26$) and 4.27 ± 0.29 mg/L ($n = 26$), respectively; a quality control sample with nominal concentration of 9.00 mg/L was used to assess the accuracy and reproducibility of the dilution step and had a measured concentration of 9.51 ± 0.13 mg/L ($n = 6$).

Data analysis

Microbiological response to each regimen was examined graphically and quantified by calculation of the area under the killing curve of \log_{10} cfu/mL from 0 to 72 h (AUBC₀₋₇₂); this area was normalized by dividing by the initial inoculum (i.e. \log_{10} cfu/mL at time zero). Changes in the PAP for ATCC 27853 were examined descriptively and quantified by calculating the area under the PAP curve (AUCPAP) normalized by the respective PAP inoculum. AUBC₀₋₇₂ and AUCPAP were calculated using the linear trapezoidal rule. Unless otherwise indicated, data are expressed as mean \pm SD.

Results

Colistin concentrations achieved for each simulated dosage regimen

For the 8, 12 and 24 hourly dosage regimens (Table 1), the mean measured concentrations immediately after dosing were 3.44 ± 0.38 ($n = 6$), 4.63 ± 0.34 ($n = 6$) and 9.30 ± 1.58 ($n = 6$) mg/L for the targeted C_{max} values of 3.0, 4.5 and 9.0 mg/L, respectively. The mean colistin $t_{1/2}$ across all experiments determined from the measured concentrations was 4.13 ± 0.49 h ($n = 18$) for the targeted value of 4 h.

Microbiological response

The time-course profiles of bacterial numbers achieved with all dosage regimens for each strain are shown in Figure 1. Substantial differences in total killing were observed between the two strains, with the clinical isolate 19056 exhibiting greater kill than ATCC 27853. All dosing regimens for both strains resulted in extensive bacterial killing to the limit of detection

(>5 \log_{10} reduction in cfu/mL) within 1–2 h of the first administration of colistin.

For ATCC 27853, regrowth after the initial administration of colistin occurred within 6 h with all regimens (Figure 1a), despite the colistin concentrations at this time (~ 1.0 , 1.6 and 3.2 mg/L for the 8, 12 and 24 hourly dosage regimens, respectively) remaining at or above the MIC of 1 mg/L. Although bacterial numbers declined after each subsequent administration of colistin, the extent of the decrease was less and generally never attained the undetectable levels observed following the first colistin dose. In addition, following the small decrease in bacterial numbers, regrowth occurred after each dose, as was observed after the first dose. At 72 h, bacterial numbers for the 24 hourly dosage regimen were virtually superimposable with those for the growth control, whereas those for the 8 and 12 hourly regimens were ~ 1.5 and ~ 0.7 \log_{10} cfu/mL below the control, respectively (Figure 1a). The general similarities in time-courses for bacterial response to each regimen are reflected in small differences in AUBC₀₋₇₂ values (Table 2).

Compared with ATCC 27853, regrowth for clinical isolate 19056 occurred more slowly after initiation of all colistin regimens and to a much lower extent (Figure 1b). It was not possible to detect regrowth with any of the regimens until 24 h. Thereafter, regrowth of bacteria was detected with each regimen. At 72 h, the cfu/mL for all regimens were >5 \log_{10} lower than for the corresponding growth control (Figure 1b). At the end of the treatment period, the maximum difference in cfu/mL between the three dosage regimens was ~ 1.25 \log_{10} units. The general similarities in time-courses for bacterial response to each regimen are again reflected in small differences in AUBC₀₋₇₂ (Table 2).

Emergence of resistance to colistin

For both ATCC 27853 (Figure 2) and the clinical isolate 19056 (data not shown), the PAP after exposure to the conditions within the *in vitro* model for 72 h, but in the absence of colistin (i.e. growth controls), closely matched those observed at time zero (baseline). At baseline or following 72 h incubation in the model for ATCC 27853, no sub-populations able to grow in the presence of 4 mg/L colistin and above were detected; for

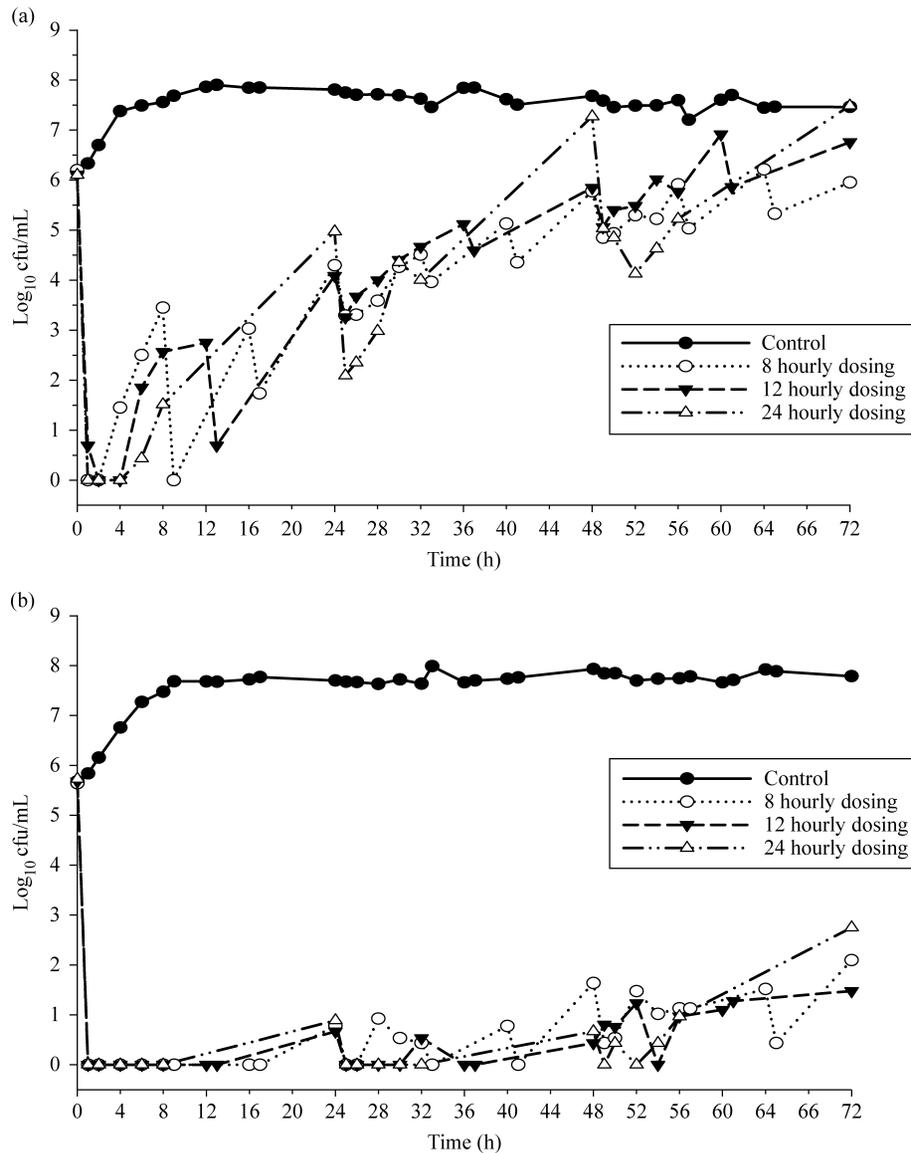


Figure 1. Microbiological response observed in the *in vitro* PK/PD model simulating the colistin pharmacokinetics ($t_{1/2}$ of 4 h) of different dosage regimens: 8 hourly dosing (C_{max} 3 mg/L), 12 hourly dosing (C_{max} 4.5 mg/L) and 24 hourly dosing (C_{max} 9 mg/L) for (a) ATCC 27853 and (b) clinical isolate 19056. Data are presented as mean values.

clinical isolate 19056, the corresponding value was 0.5 mg/L. The AUCPAPs at baseline and 72 h for clinical isolate 19056 were 0.50 ± 0.04 ($n = 3$) and 0.85 ± 0.22 ($n = 3$), respectively.

The emergence of resistance in ATCC 27853 during treatment with colistin is shown in the PAP (Figure 2); also included in the figure are the AUCPAPs. For the 8 hourly

dosage regimen, no growth was detected above 4 mg/L colistin at 48 h, whereas by 72 h, the growth was detected in the presence of colistin up to 6 mg/L (Figure 2a). For the 12 hourly dosage regimen, no growth was detected above 3 mg/L at 48 h; at 72 h, there was a very substantial change in the PAPs (Figure 2b) such that $\sim 0.14\%$ of the population was able to grow at 4 mg/L and growth was detected at 10 mg/L. For the 24

Table 2. AUCB₀₋₇₂ of the time-course of microbiological response (normalized by initial inoculum) in the *in vitro* PK/PD model

Strain	AUCB ₀₋₇₂			
	control	8 hourly dosing	12 hourly dosing	24 hourly dosing
ATCC 27853	88.4 ± 1.18	46.6 ± 1.30	50.4 ± 4.56	51.6 ± 5.75
19056	96.5 ± 0.81	8.30 ± 8.74	6.53 ± 5.77	8.43 ± 2.70

PK/PD of colistin against *Pseudomonas aeruginosa*

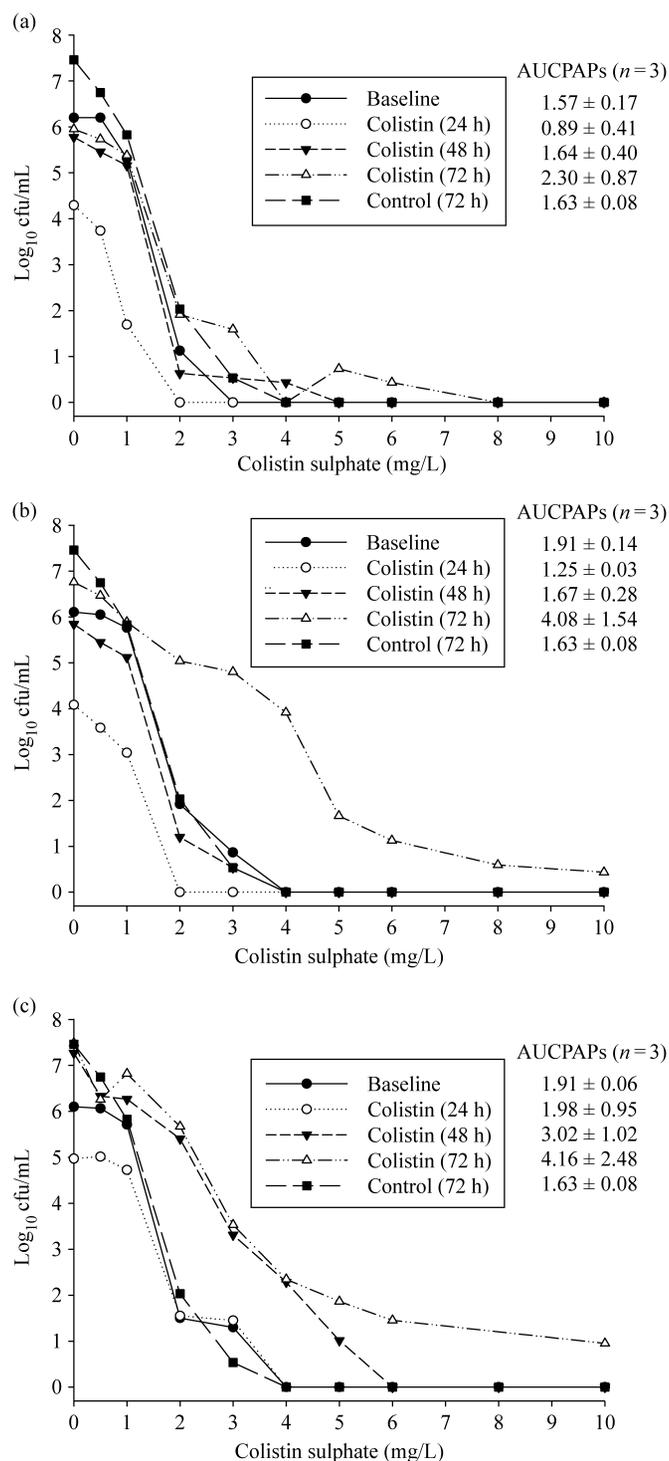


Figure 2. PAPs of ATCC 27853 in the *in vitro* PK/PD model: (a) 8 hourly dosing, (b) 12 hourly dosing and (c) 24 hourly dosing.

hourly dosage regimen, the PAP curve after 48 h moved to the right and growth was detected in the presence of 5 mg/L colistin (Figure 2c). By 72 h, there was evidence of further emergence of resistance with growth detected in the presence of 10 mg/L colistin. For clinical isolate 19056, no growth was detected in the PAP at any colistin concentration for any of the dosage regimens (data not shown).

Discussion

A lack of information on the PK and PD of colistin and CMS has led to confusion regarding the optimal dosing schedule.¹⁰ The product information for CMS recommends a maximum daily dose of 5 mg/kg/day (colistin base activity) in two to four divided doses in patients with normal renal function,^{28,29} although once-daily dosing has also been reported recently.^{26,27} Simulated regimens in the *in vitro* PK/PD model were chosen based on the PK of colistin generated from CMS in humans with normal renal function²⁰ and allowed for an unbound fraction of colistin in human plasma of approximately 0.5 (P. J. Bergen, J. Li and R. L. Nation, unpublished results). Thrice-daily dosing (8 h dosage interval, Table 1) is the regimen most commonly reported in the literature.^{7,9,23–25,35,36} A larger unit dose of colistin was administered to simulate a 12 h dosage interval²⁴ (Table 1). A 24 h dosage interval (Table 1) simulated a regimen which has recently been used clinically^{26,27} but has no corresponding recommendation for renally healthy patients in the product information.

Both bacterial strains were susceptible to colistin prior to drug exposure (MICs 1 and 0.5 mg/L for ATCC 27853 and 19056, respectively), and each dosage regimen produced colistin concentrations that exceeded the MIC for substantial percentages of the dosage interval (Table 1). With each strain, the first exposure to colistin caused rapid and extensive killing to the limit of detection (Figure 1). However, regrowth was observed with all regimens. For ATCC 27853, regrowth was detected with each regimen no later than 6 h after the initial administration of colistin, despite the concentrations at this time (~1.0, 1.6 and 3.2 mg/L for 8, 12 and 24 hourly dosage regimens, respectively) remaining at or above the MIC of 1 mg/L. For the clinical isolate 19056, the initial killing activity of colistin was more sustained, which is consistent with its lower MIC (0.5 mg/L). Simulated colistin concentrations with the 8 and 12 hourly dosage regimens remained above the MIC for this isolate throughout the treatment period (Table 1), whereas with the 24 hourly regimen, the MIC was exceeded for ~17 h (70%) of the dosage interval; the pattern of regrowth seen with this regimen, however, was not dissimilar to that of the other regimens. Indeed, regrowth with the 24 hourly regimen was detected 6 h after the 48 h dose, when colistin concentrations were significantly above the MIC (~6.4 × MIC). Thus, regrowth of the clinical isolate occurred with all regimens in the presence of colistin concentrations above the MIC, as was the case with the reference strain.

For each strain, overall bacterial killing and regrowth throughout the experimental period were generally similar among the three regimens (Figure 1 and Table 2). The AUC/MIC ratios for each strain were similar across each regimen, whereas the corresponding C_{max}/MIC and $t > MIC$ values differed substantially (Table 1). Although this study was not designed to elucidate the PK/PD index most closely related to antibacterial effect of colistin, the similar time-courses of overall bacterial numbers suggest that AUC/MIC is likely to be more important than C_{max}/MIC and $t > MIC$. The same conclusion was reached for polymyxin B from studies conducted in an *in vitro* PK/PD model with once-, twice- and thrice-daily dosing against *P. aeruginosa*.³⁷ Appropriately designed studies will be required to differentiate more definitively among the three PK/PD indices as the determinant of overall antibacterial effect.

The similarity of the time-courses of overall bacterial numbers across the regimens for a given strain may lead to the conclusion that the three regimens were equally effective. The PAP, however, provided very important information on the relative emergence of resistance across the treatment period with the three regimens. With PAP, the similarity of the profiles generated in control groups for both strains at baseline and 72 h demonstrated that incubation in the *in vitro* model in the absence of colistin did not appreciably alter the proportion of resistant sub-populations. In contrast to growth controls, the proportion of resistant sub-populations present in the reference strain following colistin administration varied with both time and regimen. We recognize that interpretation of PAP may be influenced by inoculum. As the bacterial numbers at 24 h with each regimen were substantially lower than for any other PAP samples, it is not possible to make meaningful comparison between this and other time points. In contrast, in those cases where there was a substantial change in the PAP, indicated by a 'shift to the right' and reflected by increases in AUCPAPs, the PAP inoculum was close to that of the corresponding growth controls. At 48 h, resistant sub-populations were found only with the 24 hourly dosage regimen, where growth was detected at 5 mg/L colistin (Figure 2c); the ratios of AUCPAP for the 8, 12 and 24 hourly dosage regimens to the AUCPAP for growth control at 48 h (data not shown in Figure 2) were 1.06, 1.08 and 1.95, respectively. By 72 h, resistant sub-populations were present with each regimen, but to a lesser extent with the conventional 8 h dosage interval (AUCPAP ratios of 1.41, 2.50 and 2.55 for 8, 12 and 24 h dosage intervals, respectively). Due to the low bacterial numbers present at 24, 48 and 72 h for the clinical isolate, it was not possible to use PAP to determine whether resistant sub-populations emerged.

Although the three colistin regimens led to generally similar patterns of overall bacterial numbers across the 72 h treatment period, the PAP for ATCC 27853 revealed that the emergence of resistant sub-populations increased as the dosage interval for colistin increased. Tam *et al.*³⁷ examined the antibacterial effect of polymyxin B against *P. aeruginosa* using three dosage regimens analogous to those of the present study (once, twice and thrice daily) in an *in vitro* PK/PD model. Although the extent of overall regrowth after 4 days of polymyxin B dosing was similar for each regimen, the proportion of the total population that was resistant (defined as ability to grow at $3 \times$ MIC) was substantially lower for the thrice-daily regimen when compared with the other two regimens. Although the latter observation was not commented upon,³⁷ it is in agreement with the findings of the present study with colistin, i.e. that a longer dosage interval is associated with greater emergence of resistant sub-populations.

Despite exhibiting concentration-dependent killing, colistin possesses little or no post-antibiotic effect (PAE) at clinically relevant concentrations.³⁰ In the present study, as the dosage interval increased, colistin concentrations remained above the MIC for a smaller proportion of the treatment period (Table 1). For ATCC 27853, colistin concentrations with the 8, 12 and 24 hourly dosage regimens remained above the MIC for ~80%, 72% and 53% of the 72 h treatment period, respectively. With this strain, although the time-course of bacterial numbers was generally similar among the three regimens, in the two regimens which employed the greater dosage intervals (12 and 24 h), the emergence of resistance, as revealed by PAP, was substantially greater and occurred earlier than for the conventional 8 hourly

regimen (Figure 2). It is also noteworthy that the bacterial load at 24, 48 and 72 h (the only common pre-dose sampling time across the three regimens) was greater with the 24 hourly dosage regimen than the other regimens (Figure 1a). In the absence of a substantial PAE, the emergence of resistant sub-populations appears to be favoured by extended dosage intervals leading to protracted periods of colistin concentrations below the MIC. This is an important observation given recent reports involving administration of CMS in higher, less frequent doses^{26,27} and would suggest that moves towards 24 h and other extended dosage intervals may be detrimental.

In conclusion, the emergence of resistance to colistin is of great concern given CMS is often the last available therapeutic option for treatment of infections caused by MDR Gram-negative bacteria. By simulating the PK of colistin formation in humans administered CMS, we have shown little difference in the overall pattern of bacterial killing and regrowth between three clinically relevant dosage regimens. However, we have also shown that dosing regimens incorporating higher doses of colistin administered less frequently produced greater emergence of resistance than the conventional thrice-daily regimen. This sends a strong warning about the potential negative consequences of moving prematurely to extended-interval dosing. Future studies are warranted to define the prevalence of strains in which resistance is likely to be selected by such dosage regimens. In addition, it will be important to identify the primary PK/PD index determining efficacy and preventing the emergence of resistance.

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Transparency declarations

We do not have any financial, commercial or proprietary interest in any drug, device or equipment mentioned in this paper.

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Pharmacokinetic/Pharmacodynamic Investigation of Colistin against *Pseudomonas aeruginosa* Using an *In Vitro* Model[∇]

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Colistin plays a key role in treatment of serious infections by *Pseudomonas aeruginosa*. The aims of this study were to (i) identify the pharmacokinetic/pharmacodynamic (PK/PD) index (i.e., the area under the unbound concentration-time curve to MIC ratio [$fAUC/MIC$], the unbound maximal concentration to MIC ratio [fC_{max}/MIC], or the cumulative percentage of a 24-h period that unbound concentrations exceed the MIC [$fT_{>MIC}$]) that best predicts colistin efficacy and (ii) determine the values for the predictive PK/PD index required to achieve various magnitudes of killing effect. Studies were conducted in a one-compartment *in vitro* PK/PD model for 24 h using *P. aeruginosa* ATCC 27853, PAO1, and the multidrug-resistant mucoid clinical isolate 19056 muc. Six intermittent dosing intervals, with a range of fC_{max} colistin concentrations, and two continuous infusion regimens were examined. PK/PD indices varied from 0.06 to 18 for targeted fC_{max}/MIC , 0.36 to 312 for $fAUC/MIC$, and 0 to 100% for $fT_{>MIC}$. A Hill-type model was fit to killing effect data, which were expressed as the \log_{10} ratio of the area under the CFU/ml curve for treated regimens versus control. With fC_{max} values equal to or above the MIC, rapid killing was observed following the first dose; substantial regrowth occurred by 24 h with most regimens. The overall killing effect was best correlated with $fAUC/MIC$ ($R^2 = 0.931$) compared to fC_{max}/MIC ($R^2 = 0.868$) and $fT_{>MIC}$ ($R^2 = 0.785$). The magnitudes of $fAUC/MIC$ required for 1- and 2- \log_{10} reductions in the area under the CFU/ml curve relative to growth control were 22.6 and 30.4, 27.1 and 35.7, and 5.04 and 6.81 for ATCC 27853, PAO1, and 19056 muc, respectively. The PK/PD targets identified will assist in designing optimal dosing strategies for colistin.

Globally there is a growing threat from the emergence of multidrug-resistant (MDR) microorganisms (38), especially among a number of important Gram-negative bacterial pathogens (16, 29, 38). Colistin (polymyxin E) still retains significant activity against many of these MDR Gram-negative pathogens, including *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*, which often leaves it as the only therapeutic option available (19, 26). With very few new chemical entities against Gram-negative infections in the drug development pipeline (29, 30, 38), particularly against *P. aeruginosa* (38), the use of colistin, a once-neglected antibiotic, has increased dramatically over the last 5 years (11, 26).

Colistin is available commercially as colistin sulfate (hereafter referred to as colistin) and sodium colistin methanesulfonate (CMS), which is administered parenterally. CMS is an inactive prodrug of colistin (3) and, after parenteral administration, colistin is formed *in vivo* (21, 27, 33). Despite its new-found importance in therapy, there is a dearth of information on the pharmacokinetic (PK) and pharmacodynamic (PD) properties of colistin, a situation of significant concern given

that resistance to colistin is beginning to emerge (1, 15, 18, 26, 28). Thus, the aims of the present study were to utilize an *in vitro* PK/PD model to (i) identify the PK/PD index (i.e., the area under the unbound concentration-time curve to MIC ratio [$fAUC/MIC$], the unbound maximal concentration to MIC ratio [fC_{max}/MIC], or the cumulative percentage of a 24-h period that unbound concentrations exceed the MIC [$fT_{>MIC}$]) that best predicts colistin efficacy and (ii) determine the magnitude of the predictive PK/PD index required to achieve various magnitudes of killing effect.

(Parts of the present study were presented at the 48th Annual Interscience Conference on Antimicrobial Agents and Chemotherapy [ICAAC], Washington, DC, 25 to 28 October 2008, and at the Second American Conference on Pharmacometrics, Mashantucket, CT, 4 to 7 October 2009.)

MATERIALS AND METHODS

Bacterial strains and media. Three strains of *P. aeruginosa* were used in the present study: two reference strains, ATCC 27853 and PAO1 (American Type Culture Collection, Rockville, MD), and an MDR mucoid clinical isolate, 19056 muc. The MICs of colistin, as determined by broth microdilution (6), were 1 $\mu\text{g}/\text{ml}$ for ATCC 27853 and PAO1 and 0.5 $\mu\text{g}/\text{ml}$ for 19056 muc. All MIC determinations were performed in three replicates on separate days. Storage was in tryptone soy broth (Oxoid, Basingstoke, Hampshire, England) with 20% glycerol (Ajax Finechem, Seven Hills, New South Wales, Australia) at -80°C in cryovials (Simport Plastics, Boloeil, Quebec, Canada).

Chemicals and reagents. Colistin sulfate was purchased from Sigma-Aldrich (lot 095K1048, 20,195 U/mg; St. Louis, MO). Immediately prior to each experiment, colistin stock solutions were prepared by using Milli-Q water (Millipore Australia, North Ryde, New South Wales, Australia), sterilized by filtration with a 0.22- μm -pore-size Millex-GP filter (Millipore, Bedford, MA), and then stored

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TABLE 1. Colistin dosage regimens and sampling times in the *in vitro* PK/PD model^a

Parameter	Dosage regimen ^b					
	3 h	4 h	8 h	12 h	24 h	CI
Target fC_{\max} ($\mu\text{g/ml}$)						
ATCC 27853	0.50, 1.5	1.0, 2.0, 3.5, 9.0, 18	3.0*	2.0, 4.5*, 9.0	0.20, 0.25, 0.30, 0.50, 1.0, 1.5, 3.5, 9.0*, 18	1.0, 4.5
PAO1			3.0	3.0, 9.0, 18	0.06, 0.13, 0.25, 0.50, 1.0, 2.0	1.0
19056 muc ^c			3.0*	0.06, 0.13, 0.15, 0.30, 1.0, 1.5, 4.5*	0.03, 0.06, 0.13, 0.15, 0.25, 0.35, 0.50, 1.0, 1.5, 2.0, 9.0*	
Sampling times (h) for microbiological measurements	0, 1, 2, 3, 4, 6, 7, 9, 10, 12, 13, 15, 16, 18, 19, 21, 22, 24	0, 1, 2, 4, 5, 6, 8, 9, 12, 13, 16, 17, 20, 21, 24	0, 1, 2, 4, 6, 8, 9, 16, 17, 24	0, 1, 2, 4, 6, 8, 12, 13, 24	0, 1, 2, 4, 6, 8, 24	0, 1, 2, 4, 6, 8, 12, 24

^a Dosage regimens involved intermittent administration at the dosage intervals indicated (3 to 24 h) to achieve target fC_{\max} or constant concentrations simulating continuous infusion (CI).

^b *, results taken from the first 24 h of a previous study (2).

^c For this strain, an additional 6-hourly regimen with an fC_{\max} of 1 $\mu\text{g/ml}$ was performed.

at 4°C before use; colistin is stable under these conditions (22). All other chemicals were from suppliers previously described (23).

Binding of colistin in growth medium. The binding of colistin in cation-adjusted Mueller-Hinton broth (CAMHB; Ca^{2+} at 23.0 $\mu\text{g/ml}$ and Mg^{2+} at 12.2 $\mu\text{g/ml}$; Oxoid, Hampshire, England) was measured by equilibrium dialysis using a Perspex dialysis cell unit containing two chambers (1 ml in each chamber) separated by a semipermeable membrane (Spectra/Por-2, lot 29300; Spectrum Laboratories, Rancho Dominguez, CA). Colistin (sulfate) was spiked into CAMHB (donor chamber) to achieve concentrations of 10 and 30 $\mu\text{g/ml}$ and dialyzed at 37°C against the same volume of isotonic phosphate buffer (0.067 M, pH 7.3) (acceptor chamber); samples were prepared in triplicate. Samples of CAMHB and buffer were removed from each reservoir after 24 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 colistin unbound in CAMHB (f_u) was calculated as follows: (acceptor colistin concentration)/(donor colistin concentration).

***In vitro* PK/PD model and colistin dosing regimens.** Experiments to examine the PK/PD indices driving the microbiological response to colistin were conducted over 24 h using a one-compartment *in vitro* PK/PD model (2). Briefly, the system consisted of four sealed containers (compartments), each containing 100 ml of CAMHB at 37°C and a magnetic stir bar to ensure adequate mixing. One compartment acted as a control to define growth dynamics in the absence of colistin, whereas colistin was delivered into the remaining compartments to achieve the desired intermittent injection or continuous infusion regimens (see below).

Prior to each experiment, strains were subcultured onto horse blood agar (Media Preparation Unit, The University of Melbourne, Parkville, Australia) and incubated at 35°C overnight. One colony was then selected and grown overnight in 10 ml of CAMHB, from which early-log-phase growth was obtained. A 1.0-ml aliquot of this early-log-phase bacterial suspension was inoculated into each compartment at the commencement of each experiment to yield approximately 10^6 CFU/ml.

Both intermittent and continuous infusion dosage regimens of colistin were examined. For dosage regimens involving intermittent administration of colistin, sterile drug-free CAMHB from a central reservoir was pumped through the system at a predetermined rate, displacing CAMHB from each compartment, thus simulating colistin elimination (half-life [$t_{1/2}$] = 4 h) in healthy volunteers (12) and people with cystic fibrosis (21, 34, 35). Flow rates were calibrated prior to experiments and monitored throughout to ensure the system was performing optimally. The appropriate loading dose of colistin (sulfate) was injected into each treatment compartment following bacterial inoculation to achieve the desired steady-state C_{\max} ($\cong fC_{\max}$; see Results); intermittent maintenance doses were given at appropriate intervals to achieve the same fC_{\max} as after the respective loading dose. This simulated steady-state PK with intermittent dosing. For the continuous-infusion regimens, colistin was spiked into the CAMHB within the central reservoir prior to initiation of the experiment such that all media flowing through the system (with the exception of the growth control compartment) contained a constant concentration of colistin. For both intermittent and continuous regimens, serial samples were collected aseptically, as shown

in Table 1, for viable counting and determination of colistin concentrations. Viable counting was performed by using a Whitley automatic spiral plater (WASP; Don Whitley Scientific, West Yorkshire, United Kingdom) and a ProtoCOL colony counter (Synbiosis, Cambridge, United Kingdom); the limits of counting and quantification of the procedure were 20 and 400 CFU/ml, respectively, as specified in the ProtoCOL manual.

Dosing regimens were selected to maximally differentiate among the PK/PD indices under investigation ($f\text{AUC}/\text{MIC}$, fC_{\max}/MIC , and $fT_{>\text{MIC}}$). Overall, six intermittent dosing intervals (every 3, 4, 6, 8, 12, and 24 h) were examined with fC_{\max} varied across each schedule; two continuous infusion (CI) regimens were also examined (Table 1). In all, 85 treatments across 37 different combinations of dosage frequency and fC_{\max} were examined for the three strains. PK/PD indices varied from 0.06 to 18 for targeted fC_{\max}/MIC , from 0.36 to 312 for $f\text{AUC}/\text{MIC}$, and from 0 to 100% for $fT_{>\text{MIC}}$. The range of fC_{\max} used extended to greater than that seen in humans (21) to explore the complete dose-response relationship from essentially no effect to maximum effect.

Quantification of colistin in CAMHB and buffer. Samples (250 μl) collected from the *in vitro* PK/PD model and equilibrium dialysis experiments were analyzed as previously described (2). Concentrations of colistin were measured by using HPLC with derivatization and fluorescence detection (24) with an assay range for colistin sulfate of 0.10 to 6.00 $\mu\text{g/ml}$; samples were diluted when the expected colistin concentrations were higher than the upper limit of quantification. Analysis of quality control (QC) samples with nominal concentrations of 0.40, 4.00, 9.00, and 18.00 $\mu\text{g/ml}$ (the latter two QC samples required dilution) demonstrated that the accuracy and coefficients of variation were within 15%.

Determination of predictive PK/PD index. The following PK/PD indices were determined for each dosage regimen: $f\text{AUC}/\text{MIC}$, fC_{\max}/MIC , and $fT_{>\text{MIC}}$. The area under the unbound colistin concentration-versus-time curves over 24 h ($f\text{AUC}$; $\mu\text{g} \cdot \text{h/ml}$) was determined by equation 1 (see below), where n is the number of dosing intervals in the 24-h period, and k is the elimination rate constant (0.17 h^{-1} , corresponding to a 4-h half-life). The percentage of time that unbound concentrations exceeded the MIC ($fT_{>\text{MIC}}$) was determined by equation 2. Targeted fC_{\max} , trough (fC_{\min}), and k values were used for all calculations.

$$f\text{AUC} = n \cdot (fC_{\max} - fC_{\min})/k \quad (1)$$

$$fT_{>\text{MIC}} = n \cdot \ln(fC_{\max}/\text{MIC})/k/24 \cdot 100\% \quad (2)$$

The area under the curve (AUC_{CFU}) of the time course profile of bacterial numbers (CFU/ml from 0 to 24 h) was calculated by using the linear trapezoidal rule. The killing effect (drug effect) chosen as the measure of efficacy (E) was quantified by the log ratio area method, which compensates for bacterial loss from our model (14):

$$E = \log_{10} \frac{\text{AUC}_{\text{CFU}}(\text{treatment})}{\text{AUC}_{\text{CFU}}(\text{growth control})} \quad (3)$$

The relationship between killing effect (E) and each of the three PK/PD indices was analyzed by using the Hill equation with a baseline and an inhibitory effect:

$$E = E_0 - \frac{E_{\max} \cdot x^\gamma}{EI_{50}^\gamma + x^\gamma} \quad (4)$$

where E is the observed effect, E_0 is the baseline effect in the absence of colistin, E_{\max} is the maximal effect, x is the PK/PD index under investigation, EI_{50} is the magnitude of the PK/PD index producing 50% of E_{\max} , and γ is the sigmoidicity coefficient (Hill's constant).

The parameters of equation 4 were estimated by three different approaches: (i) uniformly weighted least-squares estimation in WinNonlin Professional (version 5.2.1; Pharsight Corp., Mountain View, CA), (ii) a pooled fitting approach based on maximum-likelihood estimation in NONMEM VI (level 1.2), and (iii) nonlinear mixed-effects modeling in NONMEM VI using the first-order conditional estimation method. An additive error model on a log scale was used for approaches ii and iii. The data for all regimens were fit separately for each of the nine combinations of strains and PK/PD indices for approaches i and ii. The data for all regimens and all three strains were comodeled for approach iii for each of the PK/PD indices. Median estimates and 90% nonparametric confidence intervals (5 to 95% percentile) were determined via nonparametric bootstrapping as described previously using 1,000 replicates for each analysis of approaches ii and iii (4). For each bootstrap data set, 44 regimens were randomly chosen for strain ATCC 27853, 26 regimens were randomly chosen for 19056 muc and 15 regimens were randomly chosen for PAO1. The P values for two-sided nonparametric comparisons between PK/PD indices were computed based on the pairwise differences in objective function values between two PK/PD indices for each of the 1,000 bootstrap replicates. Determination of the PK/PD index best characterizing killing effect was assessed by the coefficient of determination (R^2), NONMEM's objective function ($-2 \cdot \log$ -likelihood), and visual inspection of the observed versus fitted effect plots.

The drug exposure ($x_{nn \log_{10} \text{ effect}}$) required for 1- or 2- \log_{10} reduction in the area under the CFU/ml curve relative to growth control (equation 5) and the drug exposure (EI_{90}) causing 90% of maximal effect (equation 6) were calculated as follows:

$$x_{nn \log_{10} \text{ reduction}} = \frac{EI_{50}}{\left(\frac{E_{\max}}{nn} - 1\right)^{\frac{1}{\gamma}}} \quad (5)$$

$$EI_{90} = \frac{EI_{50}}{\left(\frac{1}{0.9} - 1\right)^{\frac{1}{\gamma}}} \quad (6)$$

The desired extent of the \log_{10} reduction (nn) enters equation 5 as a positive number. Equation 5 yields exposure targets only if E_{\max} is larger than nn.

RESULTS

Binding of colistin in CAMHB. The fractions of colistin unbound in CAMHB (f_u) at equilibrium, with initial concentrations for colistin sulfate of 10 and 30 $\mu\text{g/ml}$, were 0.96 and 0.95, respectively, indicating practical equivalence of total and unbound concentrations.

PK validation. The mean \pm the standard deviation (SD; $n = 58$) of the absolute percentage relative differences between targeted and achieved colistin fC_{\max} concentrations as determined by high-pressure liquid chromatography (HPLC) was 9.76 ± 14.2 , and the mean of the percentage relative differences was -4.64 ± 16.7 . The observed mean $t_{1/2}$ for the simulated intermittent dosage regimens was 4.06 ± 0.46 h ($n = 47$) for the targeted value of 4 h; since the fC_{\min} for some dosage regimens was below the lower limit of quantification of the HPLC assay (0.10 $\mu\text{g/ml}$), $t_{1/2}$ was not directly measured in all experiments.

Bacterial killing of *P. aeruginosa* in the *in vitro* PK/PD model. Representative killing profiles for each strain are shown in Fig. 1. The initial inocula in control and treatment compartments (mean \pm the SD) were 6.21 ± 0.09 ($n = 15$) and 6.18 ± 0.14 ($n = 44$) \log_{10} CFU/ml for ATCC 27853, 6.39 ($n = 2$) and

6.29 ± 0.13 ($n = 15$) \log_{10} CFU/ml for PAO1, and 5.88 ± 0.41 ($n = 4$) and 6.08 ± 0.32 ($n = 26$) \log_{10} CFU/ml for 19056 muc. After 24 h, bacterial numbers in control compartments had increased to 8.06 ± 0.20 ($n = 15$) \log_{10} CFU/ml for ATCC 27853, 8.21 ($n = 2$) \log_{10} CFU/ml for PAO1, and 7.74 ± 0.07 ($n = 4$) \log_{10} CFU/ml for 19056 muc. For all strains there was early dosage-dependent killing, followed by regrowth to various extents (Fig. 1).

Relationships between killing effect and PK/PD indices.

Since the differences in the initial inocula were small (see above), we elected not to standardize AUC_{CFU} by dividing by initial inoculum when calculating the killing effect. Parameter estimates for modeling approaches i, ii, and iii were consistent for all three PK/PD indices and robust for $fAUC/MIC$ and fC_{\max}/MIC ; approach iii yielded the most robust estimates for $fT_{>MIC}$. Modeling by approach iii (Table 2) indicated that between-strain variability was largest for EI_{50} and negligible for the other parameters. The confidence intervals for EI_{50} indicated significantly lower values for strain 19056 muc. The relationships between killing effect and $fAUC/MIC$, fC_{\max}/MIC , or $fT_{>MIC}$ are shown in Fig. 2. Of the three indices, $fAUC/MIC$ best described the killing effect ($R^2 = 0.931$; Fig. 2A); the relationship between the killing effect and fC_{\max}/MIC had a lower R^2 of 0.868 (Fig. 2B). A poorer relationship existed between the killing effect and $fT_{>MIC}$, where a high degree of scatter and systematic deviations from the curve fit was observed ($R^2 = 0.785$; Fig. 2C). Median (nonparametric 90% confidence interval) objective function values from modeling approach iii were as follows: -79.6 (-111 to -54.4) for $fAUC/MIC$, -28.3 (-72.1 to 2.9) for fC_{\max}/MIC , and 16.4 (-35.9 to 47.5) for $fT_{>MIC}$. The objective function was significantly lower for $fAUC/MIC$ compared to fC_{\max}/MIC ($P = 0.050$, two-sided testing; from 1,000 nonparametric bootstrap replicates) and for $fAUC/MIC$ compared to $fT_{>MIC}$ ($P < 0.01$). Differences between fC_{\max}/MIC and $fT_{>MIC}$ were not significant ($P = 0.2$).

The magnitudes of the $fAUC/MIC$ index required for 1- and 2- \log_{10} reduction in the area under the CFU/ml curve relative to growth control for each strain are shown in Table 3. Near-maximal killing was achieved with $fAUC/MIC$ ratios of approximately 40, 50, and 9 for ATCC 27853, PAO1, and 19056 muc, respectively (Table 3 and Fig. 2A).

DISCUSSION

Colistin, which first became clinically available more than 50 years ago, was never subjected to many of the drug development procedures required of new drugs today. As a consequence, current dosage regimens for CMS/colistin are chosen empirically, and much is still to be learned about the PK, PD, and the PK/PD index that best correlates with antibacterial activity of colistin (26). Such information is important for rational design of optimal dosing strategies.

Previous animal or *in vitro* pharmacodynamic studies have reported regrowth of *P. aeruginosa* with a range of colistin (2, 17) or polymyxin B (39) dosage regimens. Similarly, this was generally observed in the present study despite unbound colistin concentrations far in excess of clinically achievable concentrations in some experiments. Even for the regimens that achieved very extensive bacterial killing after the first dose, substantially less net killing occurred after subsequent doses

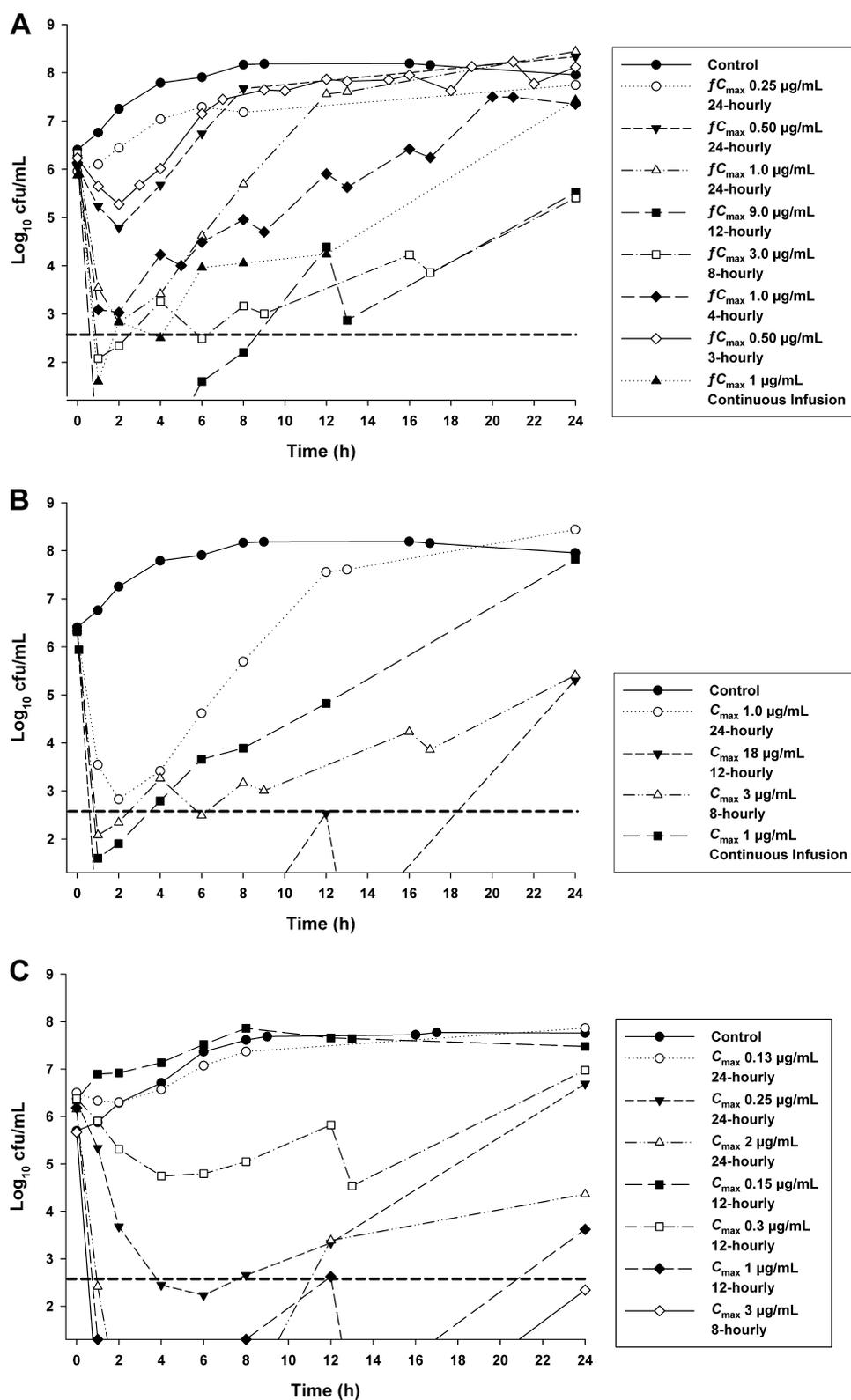


FIG. 1. Typical microbiological responses observed in the *in vitro* PK/PD model simulating the colistin pharmacokinetics of different dosage regimens using ATCC 27853 (MIC = 1 μ g/ml) (A), PAO1 (MIC = 1 μ g/ml) (B), and the MDR clinical isolate 19056 muc (MIC = 0.5 μ g/ml) (C). The y axis starts from the limit of counting, and the limit of quantification is indicated by the horizontal broken line.

TABLE 2. Median parameter estimates from 1,000 bootstrap replicates for each of the three PK/PD indices^a

PK/PD index	Strain	Median parameter estimates (90% nonparametric confidence intervals)			
		E_0	E_{max}	EI ₅₀	γ
$fAUC/MIC$	ATCC 27853	-0.232 (-0.349 to -0.139)	3.05 (2.88–3.21)	26.4 (23.8–28.9)	4.77 (3.20–7.27)
	PAO1			31.2 (28.6–35.4)	
	19056 muc			5.91 (4.60–12.5)	
fC_{max}/MIC	ATCC 27853	-0.110 (-0.319 to 0.025)	3.12 (2.81–3.38)	1.82 (1.54–2.25)	3.13 (2.08–12.2)
	PAO1			2.48 (1.74–3.27)	
	19056 muc			0.834 (0.645–1.27)	
$fT_{>MIC}$	ATCC 27853	-0.365 (-0.558 to -0.212)	3.07 (2.68–3.39)	39.6 (31.9–47.1)	2.13 (1.26–4.30)
	PAO1			68.4 (41.0–135)	
	19056 muc			13.6 (9.90–21.7)	

^a Data for all three strains were comodeled for each PK/PD index (approach iii, see Materials and Methods). Initial models with between-strain variability for all four parameters showed that the variability in E_0 , E_{max} , and γ was negligible. Since exclusion of the variability for these three parameters did not affect the objective function significantly, the final model only included between-strain variability for EI₅₀.

(Fig. 1). We have shown previously the presence of resistant subpopulations after a 72-h exposure to colistin in the same *in vitro* PK/PD model (2). We are currently developing a mechanism-based mathematical model that can describe and predict the time course of bacterial growth and killing and which incorporates the emergence of multiple bacterial populations with various colistin susceptibilities.

Three previous studies have addressed issues around the exposure-response relationships for polymyxins. Using a limited dose fractionation design, Tam et al. (39) investigated the PD of polymyxin B against *P. aeruginosa* in an *in vitro* PK/PD hollow-fiber model and suggested that activity was most likely linked to AUC/MIC. The study by Tam et al. (39), however, was not specifically designed to examine the relationship between efficacy and each PK/PD index. In a study of colistin against *P. aeruginosa* in a neutropenic mouse infection model, Keththireddy et al. (17) concluded that once-daily dosing was most effective and that the data were consistent with C_{max}/MIC being the PK/PD index most predictive of efficacy; PK data, however, were not included in that study. In neutropenic mouse thigh and lung infection models, Dudhani et al. (10) found that $fAUC/MIC$ was the index most predictive of efficacy. In the present study, we used a much larger dose fractionation design in an *in vitro* dynamic model to distinguish between PK/PD indices determining colistin efficacy. A Hill-type model was fit to the data using an area-based method whereby all CFU/ml versus time data for each regimen were taken into account. This approach allowed for a measure of the time-averaged drug effect and has been implemented in a previous investigation with vancomycin against *Staphylococcus aureus* (14). The analysis demonstrated that $fAUC/MIC$ was most closely correlated with bacterial killing (Fig. 2). Estimates of PD parameters were precise and consistent between all three estimation approaches.

In order to design dosage regimens rationally, it is necessary to know not only which PK/PD index is most predictive of bacterial killing but also the magnitude of that index needed to achieve various extents of kill (7). In the present study, respective values of $fAUC/MIC$ of ~25 and 35 for the reference strains were required to achieve 1- and 2-log reductions in the area under the CFU/ml curve relative to growth control (Table 3). These results are in extremely good agreement with those

obtained by Dudhani et al. (10) in the neutropenic mouse thigh infection model against the same two strains ($fAUC/MIC$ values of ~23 and 34 for 1- and 2-log reductions, respectively). Interestingly, the corresponding $fAUC/MIC$ values for the MDR clinical isolate were somewhat lower in our *in vitro* model compared to the neutropenic mouse thigh infection model (~6 and 7 *in vitro* compared to ~16 and 28 *in vivo* for 1- and 2-log reductions, respectively). The explanation for this difference is not known but may relate to differences in growth dynamics of this mucoid strain between *in vitro* and *in vivo* systems. We acknowledge the presence of a washout effect on bacteria with the use of open one-compartment PK/PD systems such as in our study; however, the generally good level of agreement across all strains between the present *in vitro* study and infection models involving neutropenic mice (10) is very reassuring in relation to future clinical applications of the $fAUC/MIC$ targets for colistin.

Unfortunately, it is currently not possible to compare the $fAUC/MIC$ targets from the present and other (10) preclinical studies with the $fAUC/MIC$ values achieved in infected patients receiving currently recommended CMS dosage regimens. The inability to undertake this comparison arises because recent studies have shown that colistin binding in plasma involves the acute-phase reactant α 1-acid glycoprotein (AAG), and the unbound fraction of colistin is influenced by the concentrations of both colistin and AAG (9). Since plasma AAG concentrations are influenced by pathophysiological stresses including infection (31, 40), the f_u of colistin in patients is likely to vary depending on the severity and stage of infection and magnitude of plasma colistin concentration. Although the knowledge of total plasma colistin concentrations achieved in patients is increasing (21, 25, 27, 33), there is no information on unbound plasma concentrations. As such information is forthcoming it will be possible to not only assess the ability of current CMS dosage regimens to meet the above-mentioned $fAUC/MIC$ targets but also to design optimized dosage regimens.

The use of once-daily doses of CMS has recently been reported (13, 36), presumably based upon the concentration-dependent killing of colistin observed *in vitro* (28). However, we suggest caution with this approach. First, *in vitro* data suggest that the toxicity of colistin is concentration and time de-

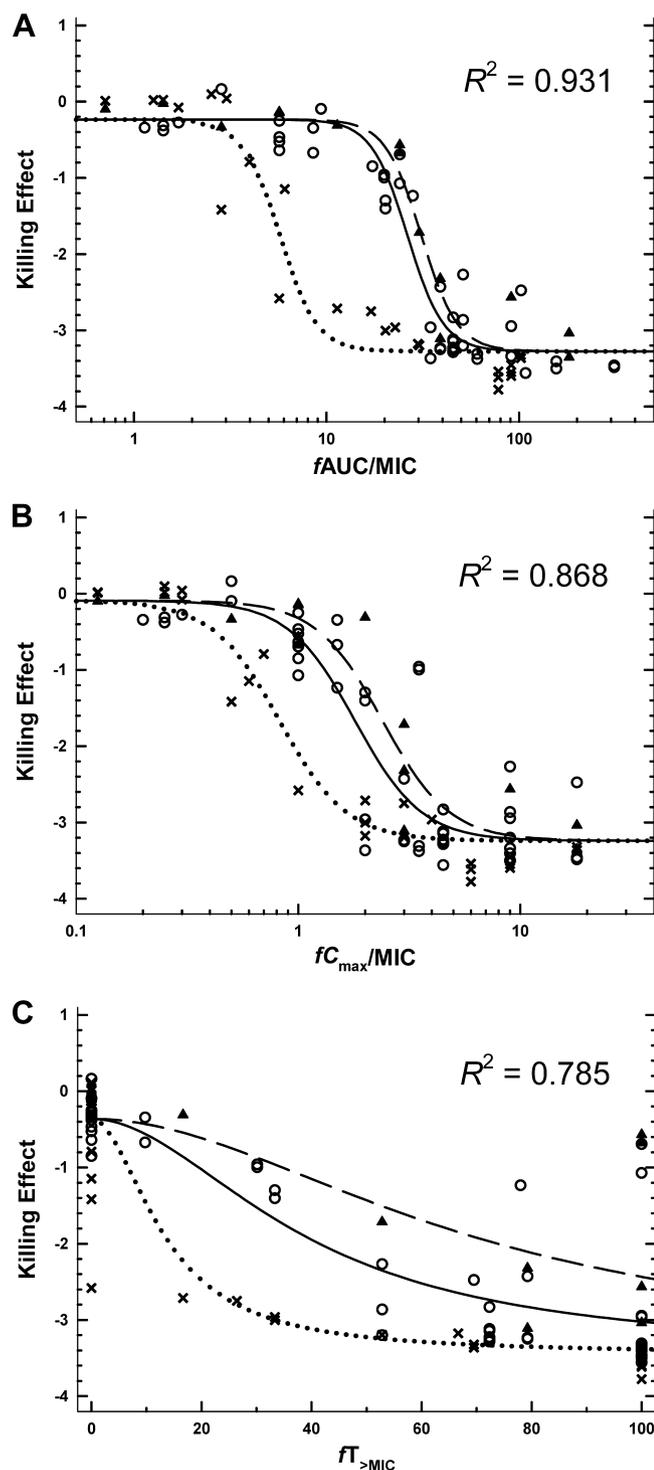


FIG. 2. Relationship between killing effect (log area ratio) against *P. aeruginosa* ATCC 27853 (solid line and open circles), PAO1 (dashed line and solid triangles), and 19056 muc (dotted line and crosses) as a function of three PK/PD indices: $fAUC/MIC$ (A), fC_{max}/MIC (B), and $fT_{>MIC}$ (C). Each data point represents the result from a single treatment run. Lines represent model-generated fits using modeling approach iii (see Materials and Methods and Table 2).

TABLE 3. Median target values from 1,000 bootstrap replicates of colistin $fAUC/MIC$ for 1- and 2- \log_{10} reductions in the area under the CFU/ml curve relative to growth control and for 90% (EI_{90}) of maximal effect

Killing effect	Median target values (90% nonparametric confidence intervals)		
	ATCC 27853	PAO1	19056 muc
1- \log_{10} reduction	22.6 (19.9–25.7)	27.1 (23.6–29.9)	5.04 (3.93–10.5)
2- \log_{10} reduction	30.4 (27.2–33.0)	35.7 (32.6–41.7)	6.81 (5.21–14.3)
EI_{90}	42.0 (35.3–52.1)	49.3 (40.8–68.5)	9.78 (6.71–20.3)

pendent (20). Moreover, greater nephrotoxicity was observed in rats with a dosage regimen mimicking once-daily dosing of CMS in humans compared to a twice-daily regimen that delivered the same daily dose (41). Second, for both colistin and polymyxin B larger, infrequent doses in *in vitro* models led to greater emergence of resistance in *P. aeruginosa* compared to lower-dose/higher-frequency regimens (2, 39). Third, colistin lacks a significant postantibiotic effect *in vitro* (28, 32), although in a brief report such a phenomenon has been suggested to occur *in vivo* (17); additional studies are needed. Given the recent emergence of resistance to colistin (1, 15, 18, 26, 28), the ability to choose regimens which not only maximize killing but also suppress or minimize the development of resistance may prove crucial in preventing this trend.

In the present study, we simulated a 4-h colistin half-life as observed in healthy volunteers (12) and people with cystic fibrosis (21, 34, 35); a longer half-life has been reported in critically ill patients (27, 33, 37). The PK/PD indices described here are specific for *P. aeruginosa* and may differ for other Gram-negative pathogens; species specific differences in the magnitude of a particular index required to achieve certain levels of killing have been demonstrated for other anti-infectives (8). In addition, *in vitro* PK/PD models lack the defense mechanisms present in patients with intact immune systems; however, they may more adequately reflect drug-related antimicrobial activity in an immunocompromised host. Finally, higher PK/PD target values (e.g., for $fAUC/MIC$) may be required for infections with a high initial inoculum (5).

To our knowledge, this is the first *in vitro* investigation specifically designed to elucidate the relationship between bacterial killing and PK/PD indices for colistin against any organism. We have demonstrated that for colistin $fAUC/MIC$ is the PK/PD index most closely correlated with the killing of *P. aeruginosa*. Our findings are in good agreement with those from recent studies in neutropenic mouse infection models. As information on the pharmacokinetics of unbound colistin in patients is obtained, the PK/PD targets reported here will assist in designing optimal dosing strategies for this increasingly important therapeutic option.

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1 **Clinically relevant plasma concentrations of colistin in combination with**
2 **imipenem enhance pharmacodynamic activity against multidrug-resistant**
3 *P. aeruginosa* at multiple inocula

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15
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24

25 **Abstract**

26

27 Use of combination antibiotic therapy may be beneficial against rapidly emerging resistance
28 in *Pseudomonas aeruginosa*. The aim of this study was to systematically investigate *in vitro*
29 bacterial killing and resistance emergence with colistin alone and in combination with
30 imipenem against multidrug-resistant (MDR) *P. aeruginosa*. Time-kill studies were
31 conducted over 48 h using 5 clinical isolates and ATCC 27853 at two inocula ($\sim 10^6$ and $\sim 10^8$
32 cfu/mL); MDR, non-MDR, and colistin-heteroresistant and -resistant strains were included.
33 Nine colistin/imipenem combinations were investigated. Microbiological response was
34 examined by log changes at 6, 24 and 48 h. Colistin combined with imipenem at clinically
35 relevant concentrations increased bacterial killing against MDR and colistin-heteroresistant
36 isolates at both inocula. Substantial improvements in activity with combinations were
37 observed across 48 h with all colistin concentrations at the low inoculum and with 4 and $16\times$
38 MIC (or 4 and 32 mg/L) colistin at the high inoculum. Combinations were additive or
39 synergistic at the 10^6 inoculum against imipenem-resistant isolates (MICs 16 and 32 mg/L) in
40 9, 11 and 12 of 18 cases (i.e., 9 combinations across 2 isolates) at 6, 24 and 48 h,
41 respectively; the corresponding values at the 10^8 inoculum were 11, 7 and 8. Against a
42 colistin-resistant strain (MIC 128 mg/L), 9 and 8 of 9 cases were additive or synergistic at 24
43 h at the 10^6 and 10^8 inocula, respectively; the corresponding values at 48 h were 5 and 7. This
44 systematic study provides important information for optimization of colistin/imipenem
45 combinations targeting both colistin-susceptible and -resistant subpopulations.

46

47

48 **Introduction**

49 The world is facing a growing threat from multidrug-resistant (MDR) Gram-negative
50 ‘superbugs’ such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella*
51 *pneumoniae* (19, 30, 50). This problem is compounded by a lack of novel antimicrobial
52 agents in the drug development pipeline for Gram-negative infections (30-31, 50), in
53 particular those caused by *P. aeruginosa* (50), and novel agents with activity against this
54 pathogen may not be available for approximately 10 years (41). This has led to the re-
55 evaluation of colistin (also known as polymyxin E), a multi-component cationic polypeptide
56 antibiotic that entered clinical use in 1959 but was largely replaced in the 1970s by
57 aminoglycosides due to concerns about the potential for nephro- and neuro-toxicity (12, 23,
58 26-27). Owing to its significant *in vitro* antibacterial activity against Gram-negative
59 ‘superbugs’, colistin is often the only therapeutic option available to treat infections by these
60 pathogens (1, 27, 35) and therefore its use has increased substantially over the last five years,
61 especially in critically-ill patients (6, 27).

62

63 It is now evident that the plasma colistin concentrations achieved in critically-ill patients with
64 the currently recommended dosage regimens are sub-optimal in a significant proportion of
65 patients (13, 43). Unfortunately, increasing the daily dose may not be an acceptable option
66 since nephrotoxicity is a dose-limiting adverse effect and occurs in 30 – 50% of patients (13,
67 16, 22). It is therefore not surprising that sub-optimal concentrations cause emergence of
68 resistance to colistin which seriously threatens colistin therapy (45, 48). *In vivo* (21, 33) and
69 *in vitro* (3-4) studies show the potential for the rapid emergence of colistin resistance with
70 monotherapy. The phenomenon of colistin heteroresistance (the presence of colistin-resistant
71 subpopulations in an isolate considered susceptible by MIC measurement) (56) has been

72 reported for *A. baumannii* (29, 56) and *K. pneumoniae* (34, 44, 54), but not yet for *P.*
73 *aeruginosa*. Heteroresistance very likely contributes to emergence of colistin resistance. The
74 aim of the present study was to systematically investigate the extent of *in vitro* bacterial
75 killing and emergence of colistin resistance with colistin alone and in combination with
76 imipenem against *P. aeruginosa*. Key aspects of this study were the use of MDR isolates with
77 varying susceptibilities to colistin and imipenem (including colistin-heteroresistant isolates
78 first identified in this study, and colistin- and imipenem-resistant strains), examination of
79 combinations of clinically relevant drug concentrations at both low and high inocula, and
80 monitoring of emergence of resistance to colistin with real-time population analysis profiles.

81

82 **Materials and Methods**

83 ***Bacterial isolates***

84 Five clinical isolates and *P. aeruginosa* ATCC 27853 (American Type Culture Collection,
85 Rockville, MD, USA) were selected to represent a mixture of colistin and imipenem
86 susceptible and resistant strains, colistin heteroresistant and non-heteroresistant strains, and
87 multidrug-resistant (MDR) and non-MDR strains. MDR was defined as diminished
88 susceptibility to at least two of the following five drug classes: antipseudomonal
89 cephalosporins, antipseudomonal carbapenems, β -lactam/ β -lactamase inhibitor combinations,
90 antipseudomonal fluoroquinolones, and aminoglycosides (40). In addition, all strains were
91 examined by PCR for the presence of genes encoding cephalosporinases and
92 carbapenemases, i.e. IMP, VIM, NDM, KPC, CTX-M, SHV, and CMY type β -lactamases
93 (47, 58). Details of the isolates are contained in Table 1. All clinical isolates were collected
94 from patients with cystic fibrosis, had different pulsed-field gel electrophoresis patterns, and
95 were considered unrelated according to the criteria established by Tenover *et al.* (53). MICs

96 to colistin and imipenem were determined for each isolate in four replicates in cation-
97 adjusted Mueller-Hinton broth (CAMHB, Ca²⁺ 23.0 mg/L, Mg²⁺ 12.2 mg/L; Oxoid,
98 Hampshire, England) via broth microdilution (10). Storage was in tryptone soy broth (Oxoid)
99 with 20% glycerol (Ajax Finechem, Seven Hills, NSW, Australia) at -80°C in cryovials
100 (Simport Plastics, Boloel, Quebec, Canada).

101

102 ***Antibiotics***

103 Colistin sulfate was purchased from Sigma-Aldrich (Lot: 109K1574, 23,251 units/mg; St
104 Louis, MO, USA). Colistin (sulfate) was employed in the current study since it is the active
105 antibacterial agent formed *in vivo* after administration of its inactive prodrug, colistin
106 methanesulfonate (CMS) (5). Imipenem was purchased from Merck Sharp and Dohme
107 (Primaxin[®], Batch: K5942; NSW, Australia). Stock solutions of each antibiotic were
108 prepared according to the respective manufacturer's instructions immediately prior to each
109 experiment to minimise loss from degradation, then sterilized by filtration with a 0.22- μ m
110 Millex-GP[®] filter (Millipore, Bedford, MA, USA).

111

112 ***Population analysis profiles***

113 The possible existence of colistin-resistant subpopulations at baseline was determined via
114 population analysis profiles (PAPs; inoculum $\sim 10^8$ cfu/mL). Colistin heteroresistance was
115 defined as a colistin-susceptible isolate (i.e., MIC ≤ 2 mg/L) in which subpopulations were
116 able to grow in the presence of >2 mg/L colistin in the PAPs. Samples of bacterial cell
117 suspension (50 μ L), appropriately diluted with saline, were spirally plated onto Mueller-
118 Hinton agar (Media Preparation Unit, The University of Melbourne, Parkville, Australia)
119 impregnated with colistin (0, 0.5, 1, 2, 3, 4, 6, 8 and 10 mg/L) using an automatic spiral plater
120 (WASP, Don Whitley Scientific, West Yorkshire, UK). Colonies were counted using a

121 ProtoCOL[®] colony counter (Synbiosis, Cambridge, UK) after 24 h of incubation (48 h for
122 plates with small colonies) at 35°C; the limit of detection was 20 cfu/mL (equivalent to 1
123 colony per plate) and limit of quantification was 400 cfu/mL (equivalent to 20 colonies per
124 plate) as specified in the ProtoCOL manual. Real-time PAPs for colistin were also conducted
125 at the end of time-kill studies (see below).

126

127 *Time-kill studies*

128 To explore the antimicrobial activity of colistin and imipenem combinations, time-kill studies
129 with each antibiotic alone or in combination were conducted on all isolates at two different
130 starting inocula ($\sim 10^6$ and $\sim 10^8$ cfu/mL). For monotherapy with colistin or imipenem, two-
131 fold multiples of the MIC (0.25 to 64× MIC) were employed for susceptible isolates. For the
132 colistin-resistant isolate (19147 n/m, MIC 128 mg/L), a single colistin concentration of 32
133 mg/L was employed. Imipenem concentrations of 1, 8 and 32 mg/L were used for imipenem-
134 resistant isolates. In combination experiments, both antibiotics were studied at concentrations
135 of 0.5, 4 and 16× MIC for susceptible isolates; for resistant isolates, concentrations of 1, 4 and
136 32 mg/L for colistin and 1, 8 and 32 mg/L for imipenem were employed. In total, nine
137 colistin/imipenem combinations were examined for each isolate at each inoculum.

138 Prior to each experiment isolates were subcultured onto horse blood agar (Media Preparation
139 Unit) and incubated at 35°C overnight. One colony was then selected and grown overnight in
140 10 mL CAMHB at 37°C from which early log-phase culture was obtained. Each antibiotic
141 was added alone or in combination to 20 mL of a log-phase broth culture of approximately
142 10^6 or 10^8 cfu/mL to yield the desired concentrations. Each 20-mL culture was placed in a
143 sterile 50-mL polypropylene tube (Greiner Bio-one) and incubated in a shaking water bath at
144 37°C. Serial samples (100 μ L) were collected aseptically for viable counting at 0, 0.5, 1, 2, 4,
145 6, 24 and 48 h, and PAPs at 48 h (see above) for all experiments involving colistin (including

146 combination arms), and viable counting only at 0, 1, 2, 4, 6, 24 and 48 h for experiments with
147 imipenem alone. Immediately after sampling and serial dilution, 50 μ L of bacterial cell
148 suspension was spirally plated onto nutrient agar with enumeration after 24 h of incubation
149 (48 h for plates with small colonies) as per PAPs above.

150

151 ***Pharmacodynamic (PD) analysis***

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

$$155 \text{ Log change} = \log_{10}(CFU_t) - \log_{10}(CFU_0)$$

156 Single antibiotic or combination regimens causing a reduction of ≥ 1 - \log_{10} cfu/mL below the
157 initial inoculum at 6, 24 or 48 h were considered active. We considered synergy to be a ≥ 2 -
158 \log_{10} lower cfu/mL for the combination relative to its most active component at the specified
159 time (42); additivity was defined as a 1 to < 2 - \log_{10} lower cfu/mL for the combination.

160

161 **Results**

162 ***Microbiological response***

163 The varying susceptibilities to colistin among the isolates are evident in the PAPs prior to
164 colistin treatment (Figure 1). Representative time-kill profiles for colistin and imipenem
165 monotherapy and combination therapy are shown in Figures 2 ($\sim 10^6$ cfu/mL inoculum) and 3
166 ($\sim 10^8$ cfu/mL inoculum). Log changes of viable cell counts at each inoculum with clinically
167 relevant colistin concentrations are presented in Tables 2 and 3. Additional time-kill and log
168 change data are presented in the electronic supplement. At the 10^6 cfu/mL inoculum,
169 regrowth was observed to various extents at 48 h with colistin monotherapy in all susceptible

170 isolates with the majority of colistin concentrations. Regrowth with imipenem monotherapy
171 was more variable and substantially less against susceptible isolates at 48 h with imipenem
172 concentrations of ≥ 4 or $8\times$ MIC, even with ESBLs present. An inoculum effect with colistin
173 monotherapy was generally observed (Figures 2 and 3, left-hand panels). The killing by
174 imipenem at the high inoculum was generally slightly slower than at the low inoculum,
175 although the extent of reductions in \log_{10} cfu/mL was comparable at both inocula (Figures 2
176 and 3).

177

178 *Isolates susceptible to both colistin and imipenem.* At the 10^6 inoculum, the addition of $0.5\times$
179 MIC colistin to imipenem (all concentrations) resulted in additivity or synergy at 6 h in 7 of 9
180 cases (i.e., 3 combinations against 3 isolates), achieving ~ 2 - to 3 - \log_{10} greater kill compared
181 to the most active equivalent monotherapy and undetectable bacterial counts in many cases
182 (Table 2 and Figure 2). By 24 or 48 h, improvements in activity with combination therapy
183 over and above the most active monotherapy (usually imipenem) were modest, particularly
184 when only clinically relevant concentrations of colistin (0.5 or $4\times$ MIC) were considered. Of
185 the 27 cases (i.e., 9 combinations against 3 isolates), 7 at 24 h and 8 at 48 h were additive or
186 synergistic, although only one case resulted in activity (i.e., ≥ 1 - \log_{10} kill) if equivalent
187 monotherapy with either drug was inactive. A similar pattern of activity was observed at the
188 10^8 inoculum. For ATCC 27853, combinations containing 4 mg/L colistin provided an
189 additional ~ 2 - \log_{10} kill to already active monotherapy at 6 h. Against all three isolates there
190 were 10 and 9 cases of additivity/synergy at 24 and 48 h, respectively, mostly involving
191 colistin at 4 or $16\times$ MIC (Table 2).

192

193 *Imipenem-resistant isolates*. For the two imipenem-resistant isolates (19271 n/m and 20891
194 n/m), there was no evidence of carbapenemase activity; most likely, an alternative resistance
195 mechanism such as the loss of major outer membrane proteins was present. At the low
196 inoculum, combination therapy resulted in substantial improvements in bacterial kill with all
197 colistin concentrations across 48 h. At 6 h additivity/synergy occurred in 9 of 18 cases (i.e., 9
198 combinations across 2 isolates), predominantly against isolate 19271 n/m, occurred with
199 combinations containing colistin at all concentrations, and produced additional reductions of
200 ~ 2 - to 6-log_{10} cfu/mL over usually active colistin monotherapy (Table 3 and Figure 2); in 5 of
201 6 cases involving 4 or $16\times$ MIC colistin against 19271 n/m, bacterial counts were reduced to
202 below the limit of detection (i.e., 20 cfu/mL). Substantial improvements in activity were also
203 present at 24 and 48 h in both isolates at all colistin concentrations. Additivity/synergy
204 occurred in 11 and 12 of 18 cases at 24 and 48 h, respectively, adding an additional ~ 1 - to 4 -
205 \log_{10} kill at 24 h and $>2.5\text{-log}_{10}$ kill at 48 h compared to that of monotherapy (Table 3 and
206 Figure 2). Interestingly, the combinations of colistin 0.5, 4 or $16\times$ MIC plus imipenem 32
207 mg/L each reduced bacterial loads to below the limit of detection at 24 h against both
208 isolates; the maximum reduction in \log_{10} cfu/mL at 24 h with colistin monotherapy at $16\times$
209 MIC was ~ 4.5 . Improvements in activity with combination therapy at the high inoculum also
210 occurred at all time points but were essentially restricted to combinations containing 4 or $16\times$
211 MIC colistin. Ten of 12 cases at 6 h containing colistin 4 (Table 3) or $16\times$ MIC (data not
212 shown) were additive or synergistic. At 24 and 48 h, the addition of imipenem at all
213 concentrations to 4 or $16\times$ MIC colistin produced additivity/synergy in over half of all cases
214 and substantially improved the activity compared with each antibiotic alone (by up to ~ 4 -
215 \log_{10} kill).

216

217 *Colistin-resistant isolate*. Bacterial killing at the 10^6 inoculum was substantially enhanced at
218 24 h, with all tested combinations being additive or synergistic and only one combination
219 (colistin 1 mg/L plus imipenem $0.5\times$ MIC) inactive (Table 3). The addition of all colistin
220 concentrations to imipenem 4 or $16\times$ MIC produced ~ 3.5 to 4.5-log_{10} kill at 24 h,
221 substantially higher than equivalent imipenem monotherapy. At 48 h all colistin
222 concentrations in combination with imipenem $4\times$ MIC were synergistic ($\sim 2\text{-}$ to 4-log_{10} kill)
223 and substantially improved activity over equivalent monotherapy. The addition of colistin 32
224 mg/L to imipenem (all concentrations) was additive or synergistic at a substantially earlier
225 time (6 h) with $\sim 1\text{-}$ to 2-log_{10} greater kill than equivalent imipenem monotherapy (overall kill
226 $\sim 3\text{-log}_{10}$ cfu/mL). At the high inoculum, additivity was achieved at 6 h with all combinations
227 containing colistin 4 mg/L (Table 3) and 32 mg/L (data not shown), and activity enhanced by
228 $\sim 1\text{-log}_{10}$ kill over imipenem monotherapy. Eight at 24 h and 7 at 48 h of 9 combinations were
229 additive or synergistic, encompassing all colistin concentrations and in many cases resulting
230 in additional reductions of $\sim 1\text{-}$ to 4-log_{10} cfu/mL over the most active monotherapy
231 (imipenem $16\times$ MIC). This enhancement of activity was particularly evident with
232 combinations containing colistin 4 or 32 mg/L, and on two occasions when combined with
233 imipenem $16\times$ MIC, no viable bacteria were detected at 48 h.

234

235 *Emergence of colistin resistance*

236 For the 4 colistin-heteroresistant isolates (Table 1), the proportion of resistant subpopulations
237 at 10^8 cfu/mL ranged from 2.2×10^{-7} to 4.7×10^{-3} (Figure 1). With colistin monotherapy
238 against the isolates susceptible to both colistin and imipenem, real-time PAPs performed at
239 48 h in the time-kill studies demonstrated an increase in colistin-resistant subpopulations at
240 both the low and high inocula with clinically relevant colistin concentrations (examples in
241 Figures 2 and 3 and the electronic supplement); no such increase was observed against isolate

242 19056 muc at the high inoculum. Against imipenem-resistant isolate 19271 n/m, colistin
243 concentrations of 0.25 to 64× MIC at the low inoculum, and 1 to 64× MIC at the high
244 inoculum resulted in nearly 100% of the remaining cells at 48 h growing in the presence of
245 10 mg/L colistin. In contrast, no increase in colistin-resistant subpopulations was observed
246 for the imipenem-resistant isolate 20891 n/m at either inoculum. Combination therapy against
247 colistin-susceptible isolates generally had little effect on the proportion of colistin-resistant
248 subpopulations at 48 h at either inoculum, the shape of the PAPs being very similar to that
249 obtained with equivalent colistin monotherapy (Figures 2 and 3).

250

251 **Discussion**

252 Although colistin has been commercially available for over 50 years (27), reliable PK/PD
253 data have only recently emerged. Population PK studies have shown that plasma colistin
254 concentrations achieved with currently recommended CMS dosage regimens are likely to be
255 suboptimal in many patients, typically generating average steady-state plasma colistin
256 concentrations of ~2 – 3 mg/L, with some patients achieving concentrations up to ~10 mg/L
257 (13, 18, 24, 28, 32, 43). Increasing the daily dose of CMS in such patients may not be an
258 option as nephrotoxicity, which occurs in ~30 – 50% of patients (16, 22), is a dose-limiting
259 adverse effect. Given these circumstances and the current last-line status of colistin therapy,
260 we chose to examine not only synergy but also additivity, as even a relatively small increase
261 in activity with combination therapy may be beneficial to patient care. As colistin is almost
262 entirely unbound in CAMHB (3), colistin concentrations of 0.5 and 4× MIC for isolates with
263 MICs ≤1 mg/L and 16× MIC for isolates with MICs of ≤0.5 mg/L (1 and 4 mg/L for colistin-
264 resistant isolates) used in our study are clinically relevant, even assuming plasma binding of
265 colistin in patients is similar to that in animals (i.e., ~50% bound) (25). All imipenem

266 concentrations employed are readily achieved in plasma after consideration of protein
267 binding (49).

268

269 As some data show that activity of both colistin (8) and imipenem (36) is attenuated at high
270 compared to low inocula, experiments were conducted at both $\sim 10^6$ and $\sim 10^8$ cfu/mL. An
271 inoculum effect was generally observed for colistin monotherapy, whereas no obvious
272 inoculum effect was present for imipenem (Figures 2 and 3). Regrowth of all isolates was
273 observed with colistin monotherapy even with colistin concentrations well above those which
274 can be safely achieved clinically. Similar regrowth with colistin (or polymyxin B)
275 monotherapy has been observed against colistin-susceptible *P. aeruginosa* both *in vitro* (4, 8,
276 15, 51) and *in vivo* (21). In *A. baumannii* and *K. pneumoniae*, regrowth following colistin
277 monotherapy has been attributed to the amplification of colistin-resistant subpopulations (11,
278 44, 52), with colistin heteroresistance reported in both species (17, 29, 44, 54). We have
279 reported here, for the first time, colistin heteroresistance in *P. aeruginosa*. The emergence of
280 colistin resistance following colistin monotherapy has previously been reported in *P.*
281 *aeruginosa* at both low and high inocula (4, 8), and a similar phenomenon was observed in
282 the present study with all isolates except 20981 n/m. While *P. aeruginosa* can undergo
283 adaptive resistance to polymyxins (14), the presence of colistin heteroresistance at baseline,
284 and the changes in PAPs after treatment, suggest regrowth following colistin monotherapy
285 may be due to amplification of pre-existing colistin-resistant subpopulations. This suggests
286 care is required with colistin monotherapy against *P. aeruginosa*, even where isolates appear
287 susceptible based on MICs.

288

289 The addition of imipenem to colistin at both inocula generally resulted in substantial
290 improvements in bacterial killing over equivalent monotherapy against MDR *P. aeruginosa*

291 isolates resistant to either antibiotic, even when ESBLs were present. The improvements in
292 activity against these isolates were observed across the 48-h duration and with all colistin
293 concentrations at the low inoculum, and 4 and 16× MIC (or 4 and 32 mg/L) colistin at the
294 high inoculum. Notably, the total reductions in log₁₀ cfu/mL achieved with combinations
295 containing lower colistin concentrations (0.5 and 4× MIC or 1 and 4 mg/L) were on many
296 occasions similar in magnitude to the reductions achieved with combinations containing 16×
297 MIC colistin, particularly at the 10⁶ inoculum (Table 3). This suggests that combinations of
298 colistin and imipenem containing clinically relevant colistin concentrations may be as
299 effective as combinations containing higher concentrations against MDR isolates when
300 resistance to either drug is present. This is an important result given that colistin-induced
301 nephrotoxicity is a dose-limiting adverse effect.

302

303 The benefits in overall antibacterial activity with the addition of imipenem to colistin were
304 less pronounced against the three isolates susceptible to both antibiotics and were generally
305 restricted to improvements in initial kill, i.e. up to 6 h (Table 2). As a proportion of patients
306 will achieve only low plasma colistin concentrations with currently recommended dosage
307 regimens (13, 43), the combination of colistin and imipenem at the commencement of
308 therapy may help to quickly reduce bacterial levels to facilitate clearance by the immune
309 system.

310

311 Previous time-kill studies have examined colistin in combination with carbapenems against
312 *P. aeruginosa* (2, 9, 38-39, 46). These studies examined colistin with imipenem, meropenem
313 or doripenem at a single inoculum (~10⁶ or 10⁷ cfu/mL), though the emergence of colistin
314 resistance was not examined (e.g. using PAPs). The present study is the first to investigate the

315 emergence of colistin resistance with colistin combination therapy. In the present
316 investigations, in cases where the combination led to extensive killing at 48 h, meaningful
317 interpretation of the PAPs was not possible (e.g. Figure 2, Panel B, colistin 4× MIC as
318 monotherapy and in combination with imipenem 4× MIC). However, when bacterial numbers
319 at 48 h were comparable, changes in PAPs with combination therapy generally mirrored
320 those observed with equivalent exposure to colistin as monotherapy. However, in both the
321 present study and previously reported studies (2, 9, 38-39, 46), static concentrations and
322 instability of carbapenems in aqueous media may have contributed to the regrowth and
323 emergence of colistin resistance at 48 h (20). Thus, it will be important to further assess the
324 utility of these combinations against a range of isolates with varying susceptibilities
325 (including heteroresistant strains) in dynamic *in vitro* models and *in vivo*.

326

327 Two possible reasons for an enhanced pharmacodynamic effect observed with the
328 combination of colistin and imipenem are subpopulation synergy and mechanistic synergy as
329 proposed previously (7). Subpopulation synergy involves one drug killing the resistant
330 subpopulation(s) of the other drug, and *vice versa*. Four of the six isolates in the present study
331 were colistin heteroresistant (Table 1), indicating the existence of colistin-resistant
332 subpopulations prior to therapy. In addition, the four imipenem-susceptible isolates were
333 imipenem heteroresistant ($MIC \leq 4$ mg/L in which subpopulations grew in the presence of >
334 4 mg/L imipenem; data not shown). Another possibility is mechanistic synergy whereby
335 colistin and imipenem acting on different cellular pathways increase the rate or extent of
336 killing of the other drug. In Gram-negative bacteria carbapenems must first gain entry into
337 the periplasmic space in order to bind to critical penicillin-binding proteins located on the
338 cytoplasmic membrane (37, 55). A number of resistance mechanisms may operate to limit the
339 concentration of carbapenems in the periplasm including the presence of carbapenem-

340 hydrolyzing enzymes and loss of outer membrane proteins (55). Polymyxins cause
341 considerable permeabilization of the outer membrane (57). It is possible that the effect of
342 colistin on membrane permeability results in substantially increased concentrations of
343 imipenem in the periplasm and improved bactericidal activity. Subpopulation and
344 mechanistic synergy are not mutually exclusive and both may operate simultaneously.
345 Further studies, including mechanism-based mathematical modelling, are ongoing to
346 investigate the mechanism(s) underpinning the enhanced pharmacodynamic activity
347 observed.

348

349 In the battle against rapidly emerging bacterial resistance in Gram-negative ‘superbugs’,
350 rational approaches to the use of combinations of existing antibiotics may be greatly
351 beneficial. To the best of our knowledge, this is the first systematic study on the PD of
352 colistin in combination with imipenem against *P. aeruginosa*, including MDR and colistin
353 heteroresistant strains, at both low and high inocula. Clinically relevant concentrations of
354 colistin in combination with imipenem substantially increased bacterial killing against MDR
355 *P. aeruginosa* at both inocula when isolates were resistant to either antibiotic. Further
356 investigations in *in vitro* pharmacodynamic systems, animal infection models and clinical
357 studies are warranted to optimize colistin/imipenem combinations targeting both colistin-
358 susceptible and -resistant subpopulations.

359

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367

368

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556

557 **Table 1:** Minimum inhibitory concentrations (MICs) of the *P. aeruginosa* isolates used in
 558 this study
 559

560

Isolate	MIC (mg/L) ^a		cephalosporinase and carbapenemase typing	MDR ^b
	Colistin	Imipenem		
ATCC 27853 ^c	1	2	negative	No
19147 n/m	128	4	IMP & CTX-M positive ^d	Yes
19056 muc	0.5	4	negative	Yes
20509 n/m ^c	0.5	1	negative	No
19271 n/m ^c	2	32	negative	Yes
20891 n/m ^c	1	16	negative	Yes

561

^a CLSI breakpoints (S, susceptible; I, intermediate; R, resistant): Colistin, S≤2 mg/L, I=4 mg/L, R≥8 mg/L; Imipenem, S≤4 mg/L, I=8 mg/L, R≥16 mg/L (10).

^b Multidrug-resistance (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 (40).

^c Colistin heteroresistant. 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 (55).

^d Contains genes encoding IMP type carbapenemase and CTX-M type extended-spectrum β-lactamase (ESBL).

562 **Table 2:** Log changes at 6, 24 or 48 h at two inocula with various clinically relevant concentrations of colistin (Col) and imipenem (Imi) against 3 isolates of
 563 *P. aeruginosa* susceptible to both antibiotics. Gray background indicates activity (a reduction of $\geq 1\text{-log}_{10}$ cfu/mL below the initial inoculum); green
 564 background indicates synergy (a $\geq 2\text{-log}_{10}$ decrease in the number of cfu/mL between the combination and its most active component); red background
 565 indicates additivity (a 1.0 to $< 2\text{-log}_{10}$ decrease in the number of cfu/mL between the combination and its most active component).

Isolate	Inoculum (cfu/mL)	Time (h)	Log change (= $\log_{10}(\text{CFU}_t) - \log_{10}(\text{CFU}_0)$)										
			Col 0.5× MIC	Col 4× MIC	Imi 0.5× MIC	Imi 4× MIC	Imi 16× MIC	Col 0.5× MIC + Imi 0.5× MIC	Col 0.5× MIC + Imi 4× MIC	Col 0.5× MIC + Imi 16× MIC	Col 4× MIC + Imi 0.5× MIC	Col 4× MIC + Imi 4× MIC	Col 4× MIC + Imi 16× MIC
ATCC 27853	~10 ⁶	6	-0.41	-5.93	-0.03	-2.77	-2.83	-2.90	-4.39	-5.95	-4.69	-5.95	-5.99
		24	+3.20	+0.06	+2.91	-3.14	-3.66	+1.71	-3.58	-5.95	-2.20	-2.68	-3.34
		48	+3.80	+1.35	+3.60	-1.06	-1.81	+3.49	-2.39	-2.90	+0.04	-1.82	-2.27
	~10 ⁸	6	+0.33	-2.48	-0.06	-2.04	-2.08	-1.00	-2.79	-2.73	-5.62	-4.94	-4.69
		24	+1.55	+0.05	+1.67	+0.14	-3.73	+1.50	-0.48	-3.42	-0.01	-3.51	-4.54
		48	+2.05	+1.65	+2.02	+1.96	-2.89	+2.04	+1.79	-3.30	+0.92	-2.25	-3.60
19056 muc	~10 ⁶	6	-2.34	-5.19	+0.45	-3.81	-5.49	-5.66	-5.69	-5.79	-5.88	-5.92	-5.75
		24	+1.63	-2.64	+2.66	-3.41	-5.49	+1.69	-4.39	-5.79	-2.62	-5.92	-5.75
		48	+3.13	+0.08	+3.27	+1.93	-5.49	+2.53	-0.27	-5.79	-0.44	-1.06	-5.75
	~10 ⁸	6	+0.11	-7.51	-0.83	-3.76	-4.22	-1.68	-4.00	-4.05	-7.96	-8.22	-7.95
		24	+0.79	-3.49	-0.01	-3.22	-5.02	+0.19	-3.46	-4.60	-2.50	-6.92	-7.95
		48	+1.42	-0.15	+0.47	+0.24	-6.08	+0.39	+0.27	-5.91	+0.08	-3.18	-7.95
20509 n/m	~10 ⁶	6	+1.47	-3.18	-1.71	-3.08	-3.83	-2.29	-5.97	-6.14	-4.34	-6.10	-5.90
		24	+3.18	+2.39	+3.10	-2.30	-3.33	+3.26	-3.04	-3.93	+1.86	-4.41	-4.30
		48	+3.61	+3.07	+3.46	-1.08	-1.43	+3.48	-1.00	-1.68	+3.21	-1.40	-1.93
	~10 ⁸	6	+0.82	-0.01	-0.81	-2.65	-2.65	-0.52	-2.86	-2.55	-1.03	-2.75	-2.53
		24	+1.62	+2.04	+1.02	-0.45	-1.48	+1.40	-0.59	-2.60	+0.81	-1.88	-3.63
		48	+1.92	+2.19	+1.25	+0.96	-1.28	+1.70	+1.58	-0.98	+1.75	+1.03	-2.37

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567

568 **Table 3:** Log changes at 6, 24, or 48 h at two inocula with various clinically relevant concentrations of colistin (Col) and imipenem (Imi) against 1 colistin-
 569 resistant, imipenem-susceptible isolate and 2 colistin-susceptible, imipenem-resistant isolates of *P. aeruginosa*. Gray background indicates activity (a
 570 reduction of $\geq 1\text{-log}_{10}$ cfu/mL below the initial inoculum); green background indicates synergy (a $\geq 2\text{-log}_{10}$ decrease in the number of cfu/mL between the
 571 combination and its most active component); red background indicates additivity (a 1.0 to $< 2\text{-log}_{10}$ decrease in the number of cfu/mL between the
 572 combination and its most active component). For colistin-resistant isolate 19147 n/m, synergy or additivity were compared with imipenem monotherapy only.

Isolate	Inoculum (cfu/mL)	Time (h)	Log change (= $\log_{10}(\text{CFU}_t) - \log_{10}(\text{CFU}_0)$)												
			Col 32 mg/L	Imi 0.5× MIC	Imi 4× MIC	Imi 16× MIC	Col 1.0 mg/L + Imi 0.5× MIC	Col 1.0 mg/L + Imi 4× MIC	Col 1.0 mg/L + Imi 16× MIC	Col 4.0 mg/L + Imi 0.5× MIC	Col 4.0 mg/L + Imi 4× MIC	Col 4.0 mg/L + Imi 16× MIC			
Col resistant, Imi susceptible	~10 ⁶	6	-0.08	-0.35	-1.44	-1.28	-1.28	-1.46	-1.77	-1.77	-1.60	-1.83			
		24	+2.16	+2.27	-0.58	-2.57	-0.71	-3.28	-4.57	-2.01	-3.89	-4.22			
		48	+2.49	+3.02	+2.50	-4.55	+2.57	-3.03	-4.46	+1.74	-1.99	-3.54			
	~10 ⁸	6	+0.04	-1.04	-1.56	-1.36	-1.83	-1.82	-1.85	-2.40	-2.70	-2.54			
		24	+0.55	+1.31	-0.33	-2.67	+0.14	-3.24	-3.38	-1.10	-4.60	-4.61			
		48	+0.86	+1.79	+1.37	-3.21	+1.18	-0.10	-3.19	+0.42	-3.82	-7.69			
	Col susceptible, Imi resistant	19271 n/m	~10 ⁶	6	-1.89	-3.32	+1.68	+1.17	-0.54	-1.32	-2.00	-4.45	-3.43	-5.77	-5.71
				24	+0.49	-2.19	+2.95	+2.89	-1.27	+0.71	+0.24	-5.75	-3.00	-5.77	-5.71
				48	+2.87	+1.83	+3.01	+2.93	+1.56	+2.88	+2.81	-2.61	+0.15	-1.52	-5.71
~10 ⁸			6	-0.05	-2.22	+0.56	+0.26	-0.95	-1.05	-0.94	-1.57	-3.13	-3.48	-4.90	
			24	+0.67	-1.13	+0.84	+0.39	-1.70	-0.37	-0.08	-2.47	-1.86	-1.76	-3.88	
			48	+0.83	-1.26	+0.83	+0.46	+0.15	+0.94	+0.98	+0.25	-1.09	+0.22	-2.30	
20891 n/m		~10 ⁶	6	+1.38	-5.95	+1.68	-0.26	-2.61	+0.35	-6.02	-5.90	-4.62	-5.90	-5.83	
			24	+2.28	-3.43	+2.53	+2.27	-3.82	+2.35	-2.90	-5.90	-3.85	-3.64	-5.83	
			48	+2.48	-1.84	+2.42	+2.28	+0.01	+2.43	+2.44	-3.82	-0.42	-3.34	-5.83	
		~10 ⁸	6	+0.21	-4.67	+0.13	-0.77	-2.50	-0.33	-1.32	-2.54	-5.76	-7.39	-7.52	
			24	+0.60	-1.12	+0.32	-0.31	-3.64	+0.54	+0.47	-2.38	-3.39	-3.47	-4.27	
			48	+0.77	-0.69	+0.55	+0.21	+0.07	+0.91	+0.84	+1.00	-3.08	-2.44	-2.89	

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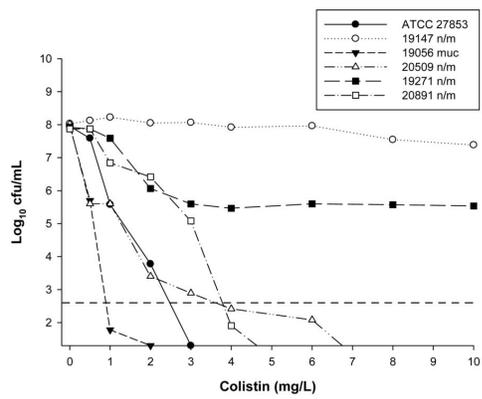
575 **Figure Captions**

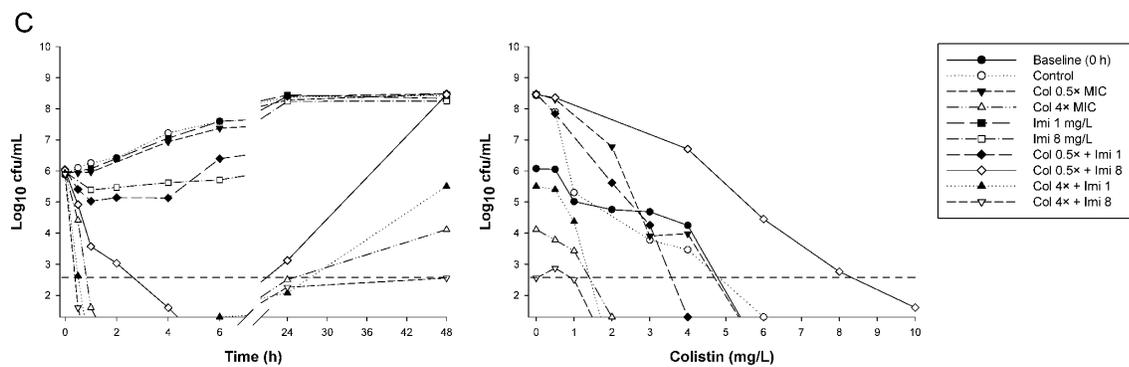
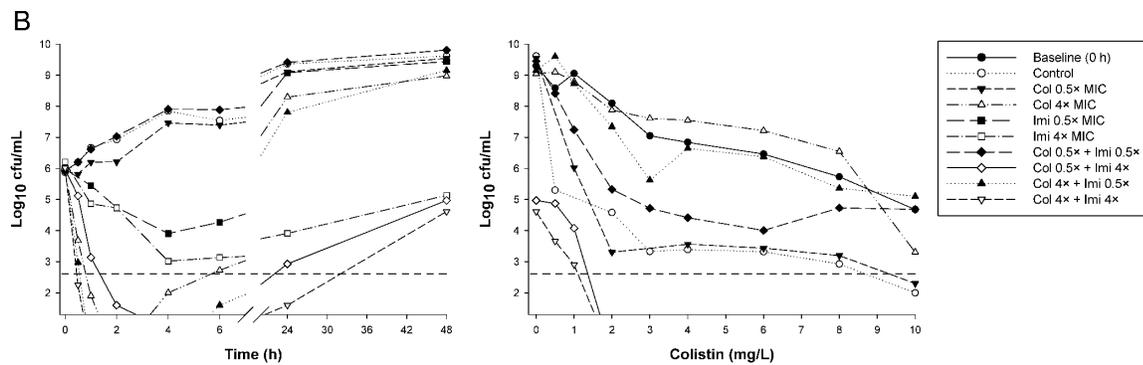
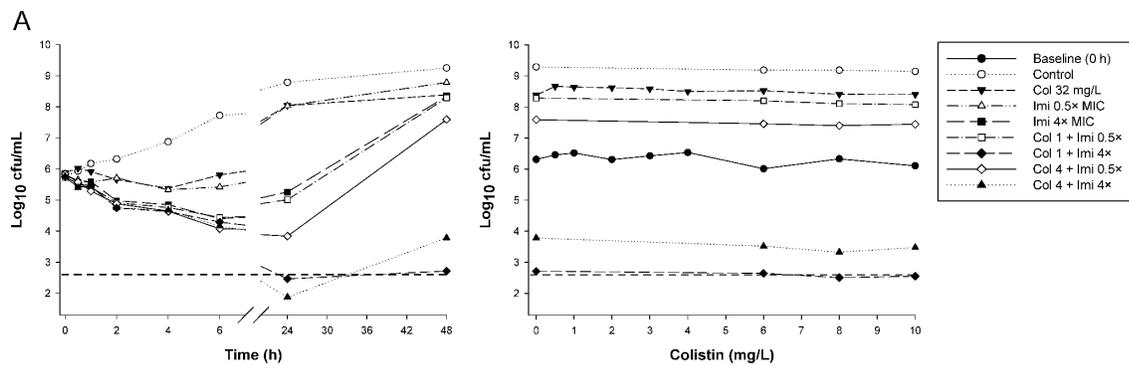
576 **Figure 1.** Baseline PAPs of the reference strain and all clinical isolates using an initial
577 inoculum of $\sim 10^8$ cfu/mL. The Y-axis starts from the limit of detection and the limit of
578 quantification (LOQ) is indicated by the horizontal broken line.

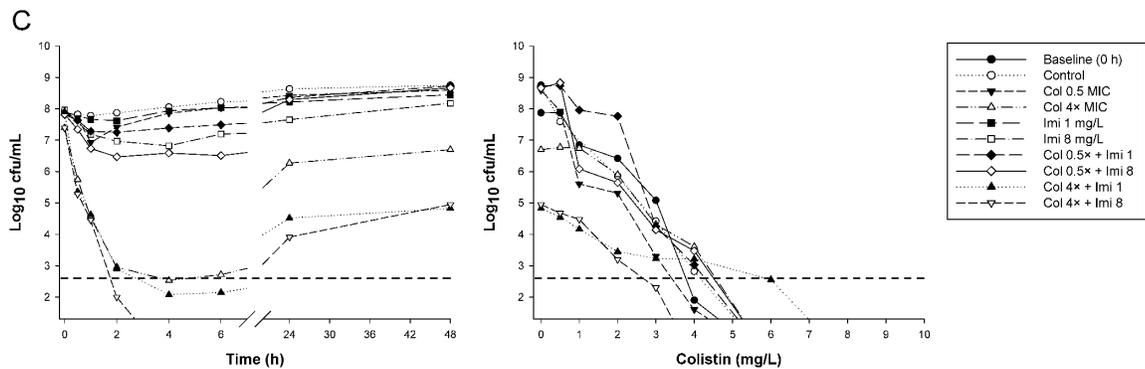
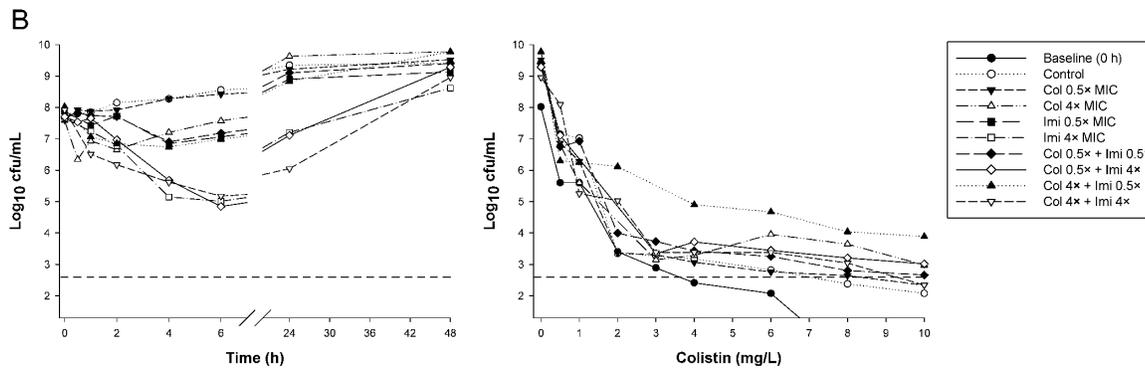
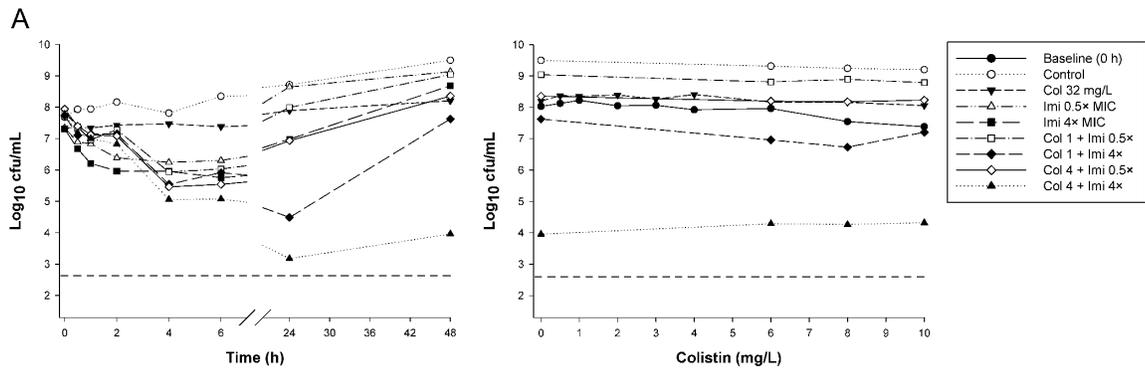
579 **Figure 2.** Representative time-kill curves (left-hand panels) with various clinically relevant
580 concentrations of colistin and imipenem alone and in combination at an inoculum of $\sim 10^6$
581 cfu/mL against (A) 19147 n/m (colistin-resistant, imipenem-susceptible MDR), (B) 20509
582 n/m (colistin- and imipenem-susceptible non-MDR) and (C) 20891 n/m (colistin-susceptible,
583 imipenem-resistant MDR). Right-hand panels show the respective PAPs at baseline (0 h) and
584 after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy or
585 neither antibiotic (control). The Y-axis starts from the limit of detection and the limit of
586 quantification (LOQ) is indicated by the horizontal broken line.

587 **Figure 3.** Representative time-kill curves (left-hand panels) with various clinically relevant
588 concentrations of colistin and imipenem alone and in combination at an inoculum of $\sim 10^8$
589 cfu/mL against (A) 19147 n/m (colistin-resistant, imipenem-susceptible MDR), (B) 20509
590 n/m (colistin- and imipenem-susceptible nonMDR) and (C) 20891 n/m (colistin-susceptible,
591 imipenem-resistant MDR). Right-hand panels show the respective PAPs at baseline (0 h) and
592 after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy or
593 neither antibiotic (control). The Y-axis starts from the limit of detection and the limit of
594 quantification (LOQ) is indicated by the horizontal broken line.

595







Supplement – Bergen *et al.*, Clinically relevant plasma concentrations of colistin in combination with imipenem enhance PD activity against MDR *P. aeruginosa* at multiple inocula

Table S1: Log changes at 6, 24 or 48 h at two inocula with colistin (Col) and imipenem (Imi) against 3 isolates of *P. aeruginosa* susceptible to both antibiotics. Gray background indicates activity (a reduction of $\geq 1\text{-log}_{10}$ cfu/mL below the initial inoculum); green background indicates synergy (a $\geq 2\text{-log}_{10}$ decrease in the number of cfu/mL between the combination and its most active component); red background indicates additivity (a 1.0 to $< 2\text{-log}_{10}$ decrease in the number of cfu/mL between the combination and its most active component).

Isolate	Inoculum (cfu/mL)	Time (h)	Log change (= $\log_{10}(\text{CFU}_t) - \log_{10}(\text{CFU}_0)$)														
			Col 0.5× MIC	Col 4× MIC	Col 16× MIC*	Imi 0.5× MIC	Imi 4× MIC	Imi 16× MIC	Col 0.5× MIC + Imi 0.5× MIC	Col 0.5× MIC + Imi 4× MIC	Col 0.5× MIC + Imi 16× MIC	Col 4× MIC + Imi 0.5× MIC	Col 4× MIC + Imi 4× MIC	Col 4× MIC + Imi 16× MIC	Col 16× MIC + Imi 0.5× MIC	Col 16× MIC + Imi 4× MIC	Col 16× MIC + Imi 16× MIC
ATCC 27853	~10 ⁶	6	-0.41	-5.93	-6.03	-0.03	-2.77	-2.83	-2.90	-4.39	-5.95	-4.69	-5.95	-5.99	-5.98	-5.97	-6.01
		24	+3.20	+0.06	-1.23	+2.91	-3.14	-3.66	+1.71	-3.58	-5.95	-2.20	-2.68	-3.34	-0.35	-1.65	-6.01
		48	+3.80	+1.35	+0.39	+3.60	-1.06	-1.81	+3.49	-2.39	-2.90	+0.04	-1.82	-2.27	+0.90	-0.98	-6.01
	~10 ⁸	6	+0.33	-2.48	-7.68	-0.06	-2.04	-2.08	-1.00	-2.79	-2.73	-5.62	-4.94	-4.69	-7.76	-7.78	-7.81
		24	+1.55	+0.05	-3.34	+1.67	+0.14	-3.73	+1.50	-0.48	-3.42	-0.01	-3.51	-4.54	-2.83	-7.78	-7.81
		48	+2.05	+1.65	-1.97	+2.02	+1.96	-2.89	+2.04	+1.79	-3.30	+0.92	-2.25	-3.60	-1.46	-7.78	-7.81

*For ATCC 27853, Col 8× MIC used instead of 16× MIC at the 10⁶ cfu/mL inoculum only.

Table S1. (Continued)

Isolate	Inoculum (cfu/mL)	Time (h)	Log change (= log ₁₀ (CFU _t) - log ₁₀ (CFU ₀))															
			Col 0.5× MIC	Col 4× MIC	Col 16× MIC*	Imi 0.5× MIC	Imi 4× MIC	Imi 16× MIC	Col 0.5× MIC + Imi 0.5× MIC	Col 0.5× MIC + Imi 4× MIC	Col 0.5× MIC + Imi 16× MIC	Col 4× MIC + Imi 0.5× MIC	Col 4× MIC + Imi 4× MIC	Col 4× MIC + Imi 16× MIC	Col 16× MIC + Imi 0.5× MIC	Col 16× MIC + Imi 4× MIC	Col 16× MIC + Imi 16× MIC	
19056 muc	~10 ⁶	6	-2.34	-5.19	-5.16	+0.45	-3.81	-5.49	-5.66	-5.69	-5.79	-5.88	-5.92	-5.75	-5.82	-5.89	-5.88	
		24	+1.63	-2.64	-5.16	+2.66	-3.41	-5.49	+1.69	-4.39	-5.79	-2.62	-5.92	-5.75	-5.82	-5.89	-5.88	
		48	+3.13	+0.08	-0.37	+3.27	+1.93	-5.49	+2.53	-0.27	-5.79	-0.44	-1.06	-5.75	-5.82	-5.89	-5.88	
	~10 ⁸	6	+0.11	-7.51	-7.94	-0.83	-3.76	-4.22	-1.68	-4.00	-4.05	-7.96	-8.22	-7.95	-7.94	-7.91	-7.76	
		24	+0.79	-3.49	-7.94	-0.01	-3.22	-5.02	+0.19	-3.46	-4.60	-2.50	-6.92	-7.95	-7.94	-7.91	-7.76	
		48	+1.42	-0.15	-5.03	+0.47	+0.24	-6.08	+0.39	+0.27	-5.91	+0.08	-3.18	-7.95	-4.00	-4.45	-7.76	
	20509 n/m	~10 ⁶	6	+1.47	-3.18	-5.89	-1.71	-3.08	-3.83	-2.29	-5.97	-6.14	-4.34	-6.10	-5.90	-5.95	-6.05	-6.05
			24	+3.18	+2.39	-0.94	+3.10	-2.30	-3.33	+3.26	-3.04	-3.93	+1.86	-4.41	-4.30	-3.35	-6.05	-6.05
			48	+3.61	+3.07	+0.81	+3.46	-1.08	-1.43	+3.48	-1.00	-1.68	+3.21	-1.40	-1.93	-1.80	-4.27	-3.58
~10 ⁸		6	+0.82	-0.01	-3.41	-0.81	-2.65	-2.65	-0.52	-2.86	-2.55	-1.03	-2.75	-2.53	-3.50	-4.94	-7.94	
		24	+1.62	+2.04	-0.28	+1.02	-0.45	-1.48	+1.40	-0.59	-2.60	+0.81	-1.88	-3.63	-1.58	-3.71	-4.49	
		48	+1.92	+2.19	+1.89	+1.25	+0.96	-1.28	+1.70	+1.58	-0.98	+1.75	+1.03	-2.37	+1.03	-1.42	-3.22	

Table S2: Log changes at 6, 24, or 48 h at two inocula with colistin (Col) and imipenem (Imi) against a colistin-resistant, imipenem-susceptible isolate and two colistin-susceptible, imipenem-resistant isolates of *P. aeruginosa*. Gray background indicates activity (a reduction of $\geq 1\text{-log}_{10}$ cfu/mL below the initial inoculum); green background indicates synergy (a $\geq 2\text{-log}_{10}$ decrease in the number of cfu/mL between the combination and its most active component); red background indicates additivity (a 1.0 to $< 2\text{-log}_{10}$ decrease in the number of cfu/mL between the combination and its most active component). For colistin-resistant isolate 19147 n/m, synergy or additivity were compared with imipenem monotherapy only.

Isolate	Inoculum (cfu/mL)	Time (h)	Log change (= $\log_{10}(\text{CFU}_t) - \log_{10}(\text{CFU}_0)$)												
			Col 32 mg/L	Imi 0.5× MIC	Imi 4× MIC	Imi 16× MIC	Col 1.0 mg/L + Imi 0.5× MIC	Col 1.0 mg/L + Imi 4× MIC	Col 1.0 mg/L + Imi 16× MIC	Col 4.0 mg/L + Imi 0.5× MIC	Col 4.0 mg/L + Imi 4× MIC	Col 4.0 mg/L + Imi 16× MIC	Col 32 mg/L + Imi 0.5× MIC	Col 32 mg/L + Imi 4× MIC	Col 32 mg/L + Imi 16× MIC
Col resistant, Imi susceptible	~10 ⁶	6	-0.08	-0.35	-1.44	-1.28	-1.28	-1.46	-1.77	-1.77	-1.60	-1.83	-2.81	-2.79	-3.15
		24	+2.16	+2.27	-0.58	-2.57	-0.71	-3.28	-4.57	-2.01	-3.89	-4.22	-1.95	-3.95	-4.26
		48	+2.49	+3.02	+2.50	-4.55	+2.57	-3.03	-4.46	+1.74	-1.99	-3.54	+1.58	-3.72	-4.21
	~10 ⁸	6	+0.04	-1.04	-1.56	-1.36	-1.83	-1.82	-1.85	-2.40	-2.70	-2.54	-2.54	-2.65	-2.79
		24	+0.55	+1.31	-0.33	-2.67	+0.14	-3.24	-3.38	-1.10	-4.60	-4.61	-1.02	-5.90	-6.11
		48	+0.86	+1.79	+1.37	-3.21	+1.18	-0.10	-3.19	+0.42	-3.82	-7.69	-0.02	-3.09	-7.71

Table S2. (Continued)

Isolate	Inoculum (cfu/mL)	Time (h)	Log change (= $\log_{10}(\text{CFU}_t) - \log_{10}(\text{CFU}_0)$)															
			Col 0.5x MIC	Col 4x MIC	Col 16x MIC*	Imi 1.0 mg/L	Imi 8.0 mg/L	Imi 32 mg/L	Col 0.5x MIC	Col 0.5x MIC	Col 0.5x MIC	Col 4x MIC	Col 4x MIC	Col 4x MIC	Col 4x MIC	Col 16x MIC	Col 16x MIC	Col 16x MIC
Col susceptible, Imi resistant	~10 ⁶	6	-1.89	-3.32	-3.88	+1.68	+1.17	-0.54	-1.32	-2.00	-4.45	-3.43	-5.77	-5.71	-5.70	-5.77	-5.75	
		24	+0.49	-2.19	-3.68	+2.95	+2.89	-1.27	+0.71	+0.24	-5.75	-3.00	-5.77	-5.71	-3.92	-5.77	-5.75	
		48	+2.87	+1.83	-1.29	+3.01	+2.93	+1.56	+2.88	+2.81	-2.61	+0.15	-1.52	-5.71	-2.89	-5.77	-5.75	
		~10 ⁸	6	-0.05	-2.22	-3.18	+0.56	+0.26	-0.95	-1.05	-0.94	-1.57	-3.13	-3.48	-4.90	-4.81	-4.97	-5.36
			24	+0.67	-1.13	-2.95	+0.84	+0.39	-1.70	-0.37	-0.08	-2.47	-1.86	-1.76	-3.88	-2.52	-3.66	-6.22
			48	+0.83	-1.26	-0.46	+0.83	+0.46	+0.15	+0.94	+0.98	+0.25	-1.09	+0.22	-2.30	-0.01	-3.14	-4.24
	20891 n/m	~10 ⁶	6	+1.38	-5.95	-5.97	+1.68	-0.26	-2.61	+0.35	-6.02	-5.90	-4.62	-5.90	-5.83	-5.92	-5.96	-5.98
			24	+2.28	-3.43	-4.57	+2.53	+2.27	-3.82	+2.35	-2.90	-5.90	-3.85	-3.64	-5.83	-5.92	-5.96	-5.98
			48	+2.48	-1.84	-3.20	+2.42	+2.28	+0.01	+2.43	+2.44	-3.82	-0.42	-3.34	-5.83	-3.77	-5.96	-5.98
		~10 ⁸	6	+0.21	-4.67	-5.50	+0.13	-0.77	-2.50	-0.33	-1.32	-2.54	-5.76	-7.39	-7.52	-5.97	-7.35	-7.52
			24	+0.60	-1.12	-2.65	+0.32	-0.31	-3.64	+0.54	+0.47	-2.38	-3.39	-3.47	-4.27	-4.60	-5.35	-4.83
			48	+0.77	-0.69	-2.61	+0.55	+0.21	+0.07	+0.91	+0.84	+1.00	-3.08	-2.44	-2.89	-3.65	-3.92	-3.58

Time-kill plots:

Figure S1: Time-kill curves for ATCC 27853 at an inoculum of $\sim 10^6$ cfu/mL with (A) colistin alone, (B) imipenem alone, and (C) in combination. Panel (D) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the limit of quantification (LOQ) is indicated by the horizontal broken line.

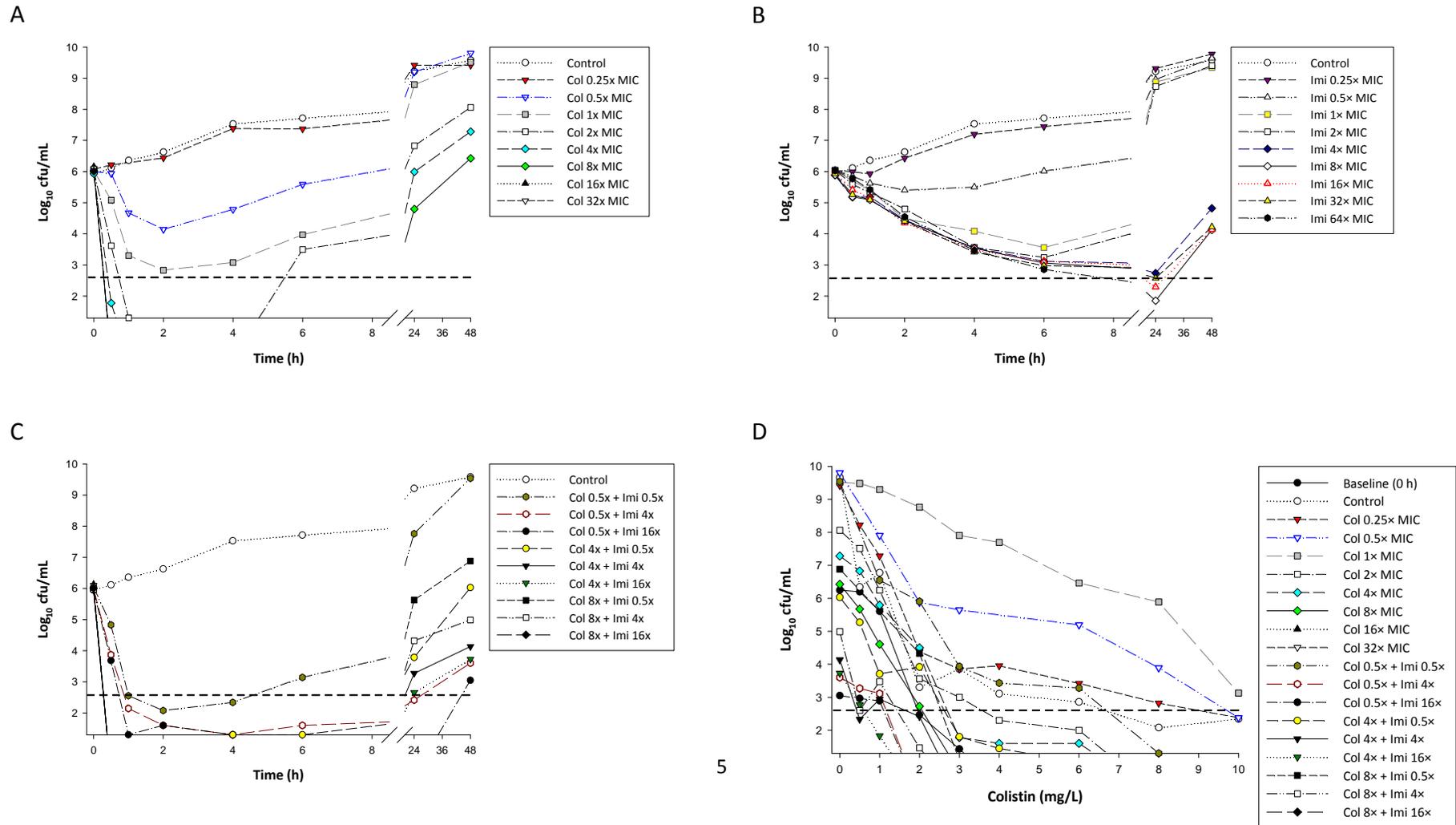


Figure S2: Time-kill curves for ATCC 27853 at an inoculum of $\sim 10^8$ cfu/mL with (A) colistin alone, (B) imipenem alone, and (C) in combination. Panel (D) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the limit of quantification (LOQ) is indicated by the horizontal broken line.

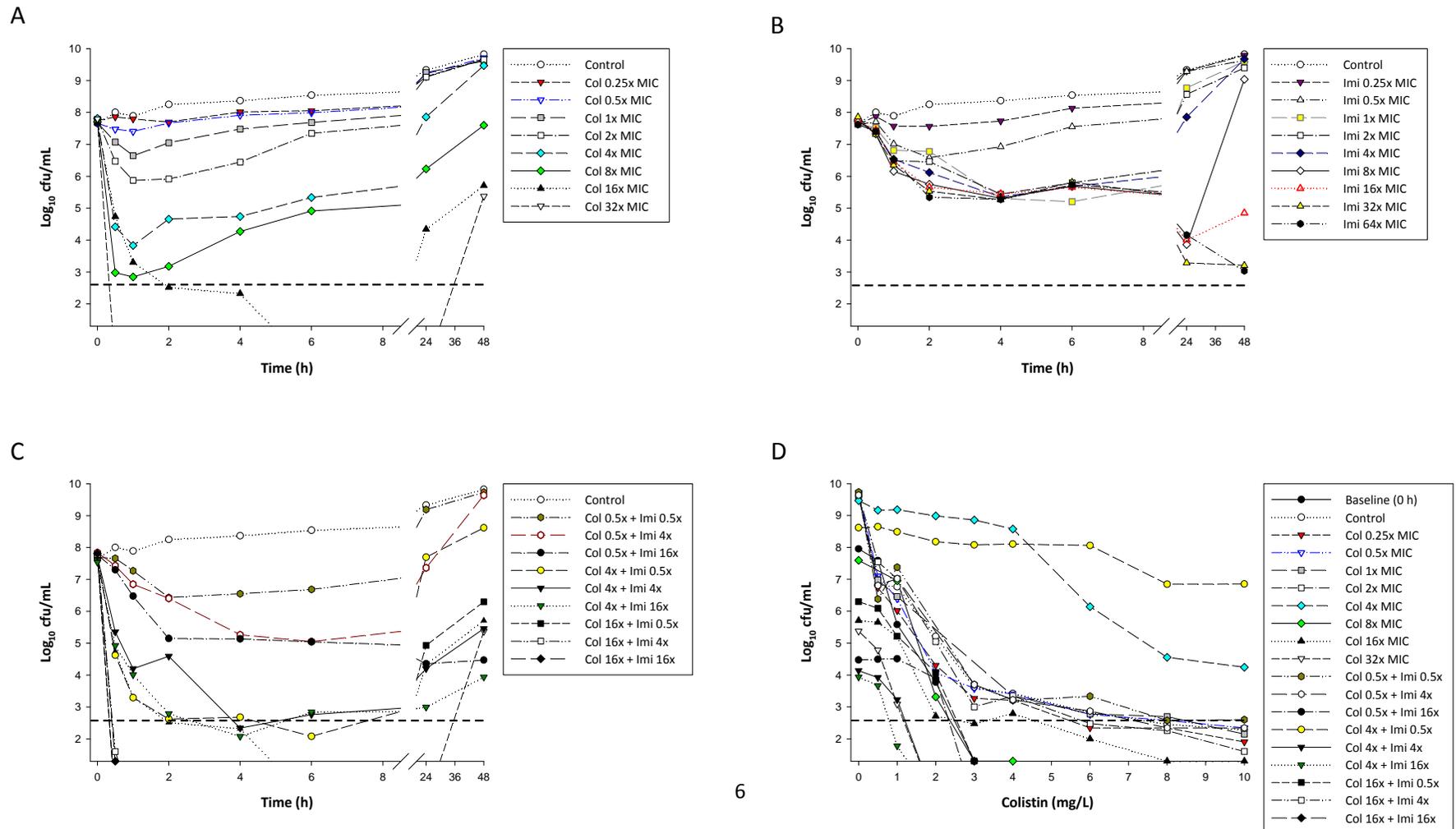


Figure S3: Time-kill curves for 19147 n/m at an inoculum of $\sim 10^6$ cfu/mL with (A) colistin and imipenem alone, and (B) in combination. Panel (C) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the limit of quantification (LOQ) is indicated by the horizontal broken line.

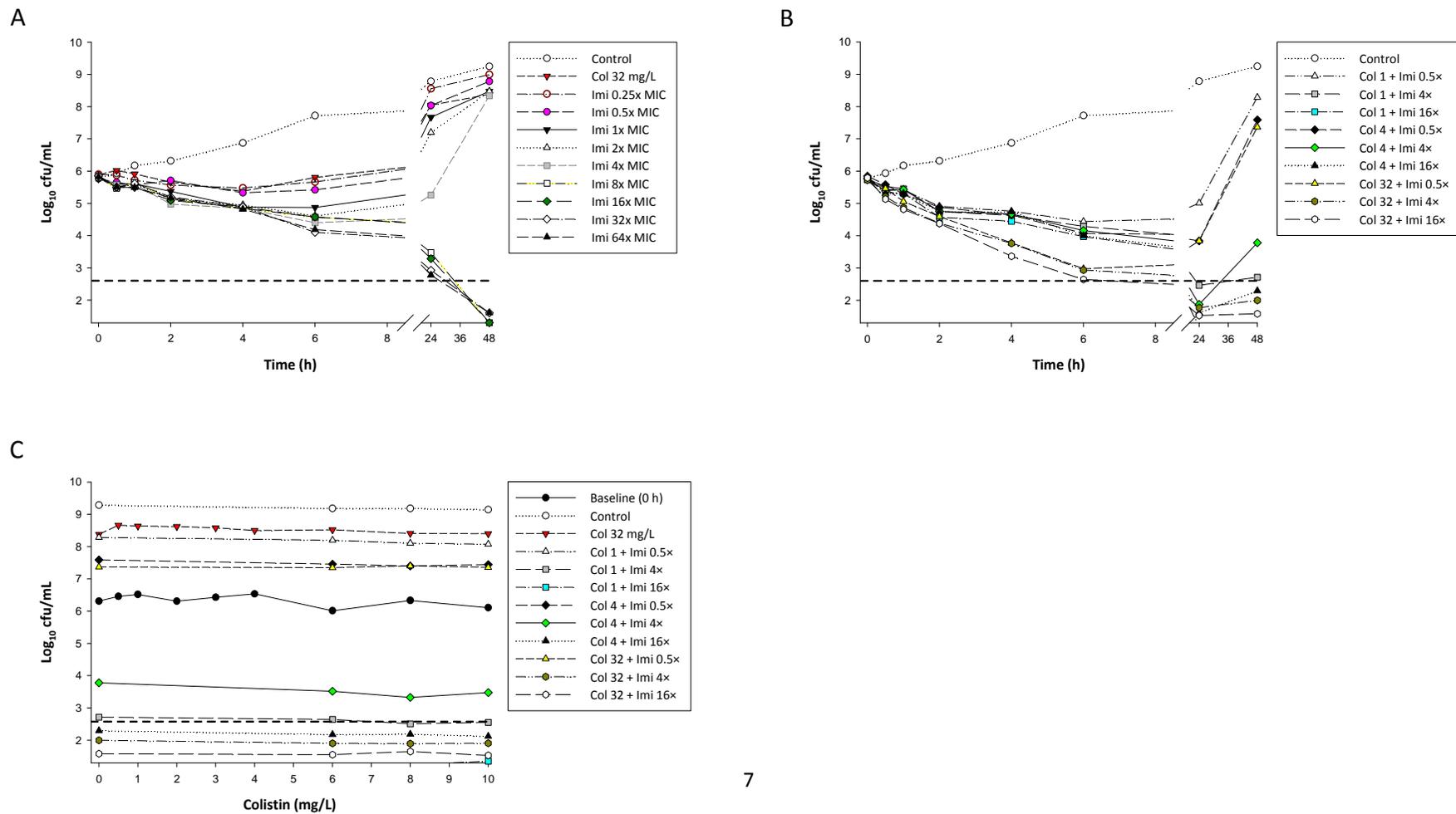


Figure S4: Time-kill curves for 19147 n/m at an inoculum of $\sim 10^8$ cfu/mL with (A) colistin and imipenem alone, and (B) in combination. Panel (C) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the limit of quantification (LOQ) is indicated by the horizontal broken line.

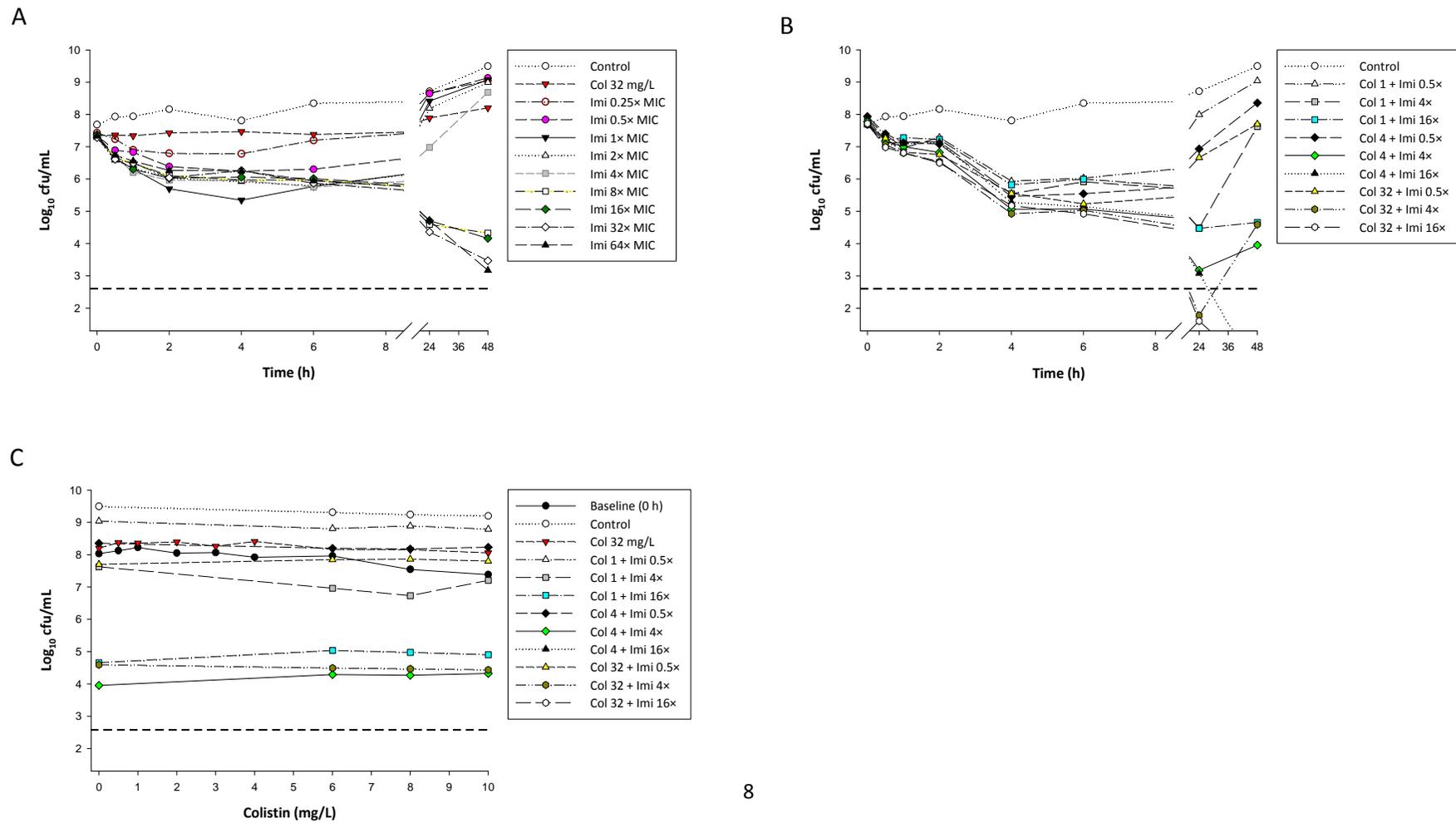


Figure S5: Time-kill curves for 19056 muc at an inoculum of $\sim 10^6$ cfu/mL with (A) colistin alone, (B) imipenem alone, and (C) in combination. Panel (D) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the limit of quantification (LOQ) is indicated by the horizontal broken line.

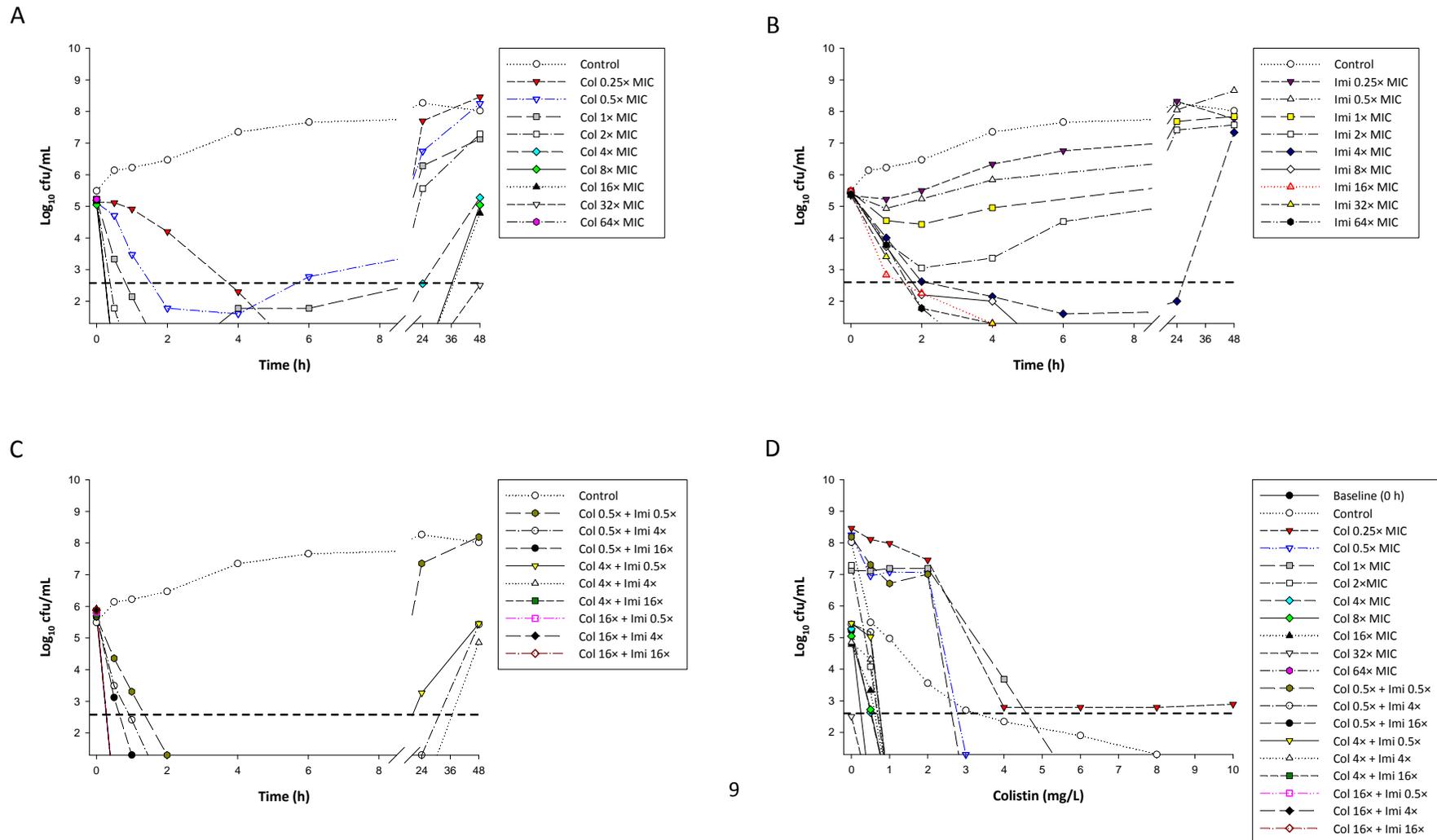


Figure S6: Time-kill curves for 19056 muc at an inoculum of $\sim 10^8$ cfu/mL with (A) colistin alone, (B) imipenem alone, and (C) in combination. Panel (D) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the limit of quantification (LOQ) is indicated by the horizontal broken line.

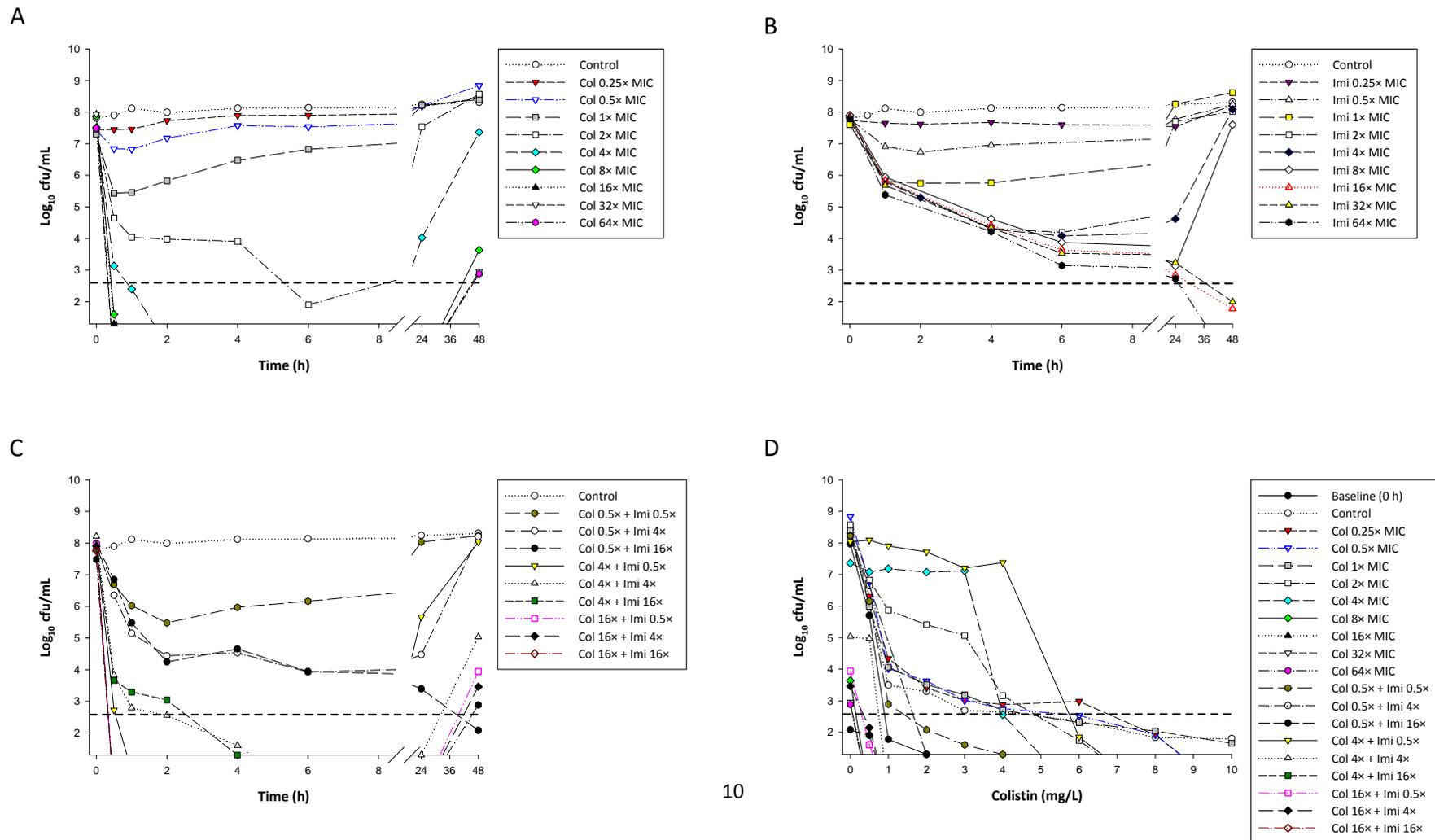


Figure S7: Time-kill curves for 20509 n/m at an inoculum of $\sim 10^6$ cfu/mL with (A) colistin alone, (B) imipenem alone, and (C) in combination. Panel (D) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the limit of quantification (LOQ) is indicated by the horizontal broken line.

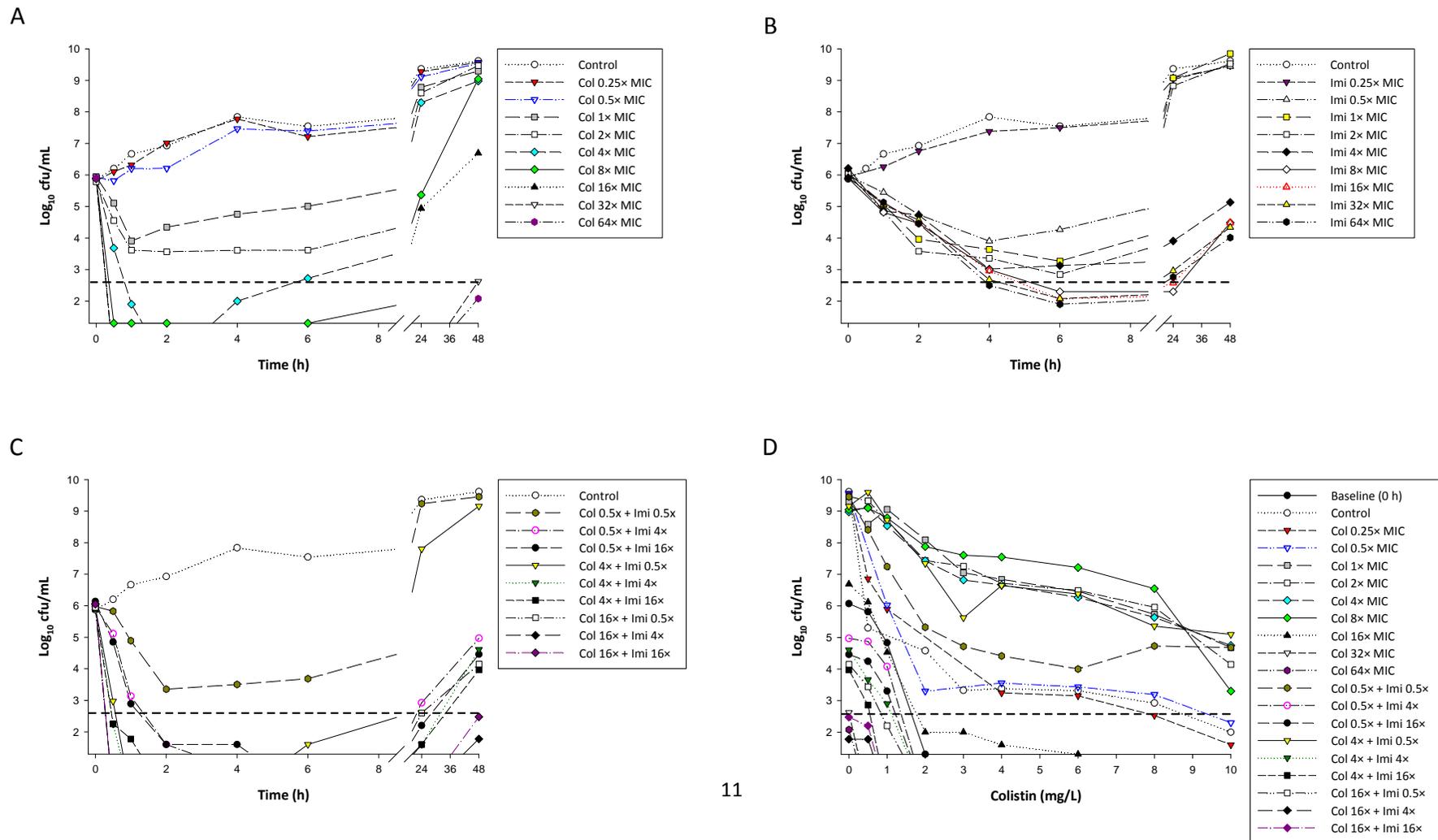


Figure S8: Time-kill curves for 20509 n/m at an inoculum of $\sim 10^8$ cfu/mL with (A) colistin alone, (B) imipenem alone, and (C) in combination. Panel (D) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the limit of quantification (LOQ) is indicated by the horizontal broken line.

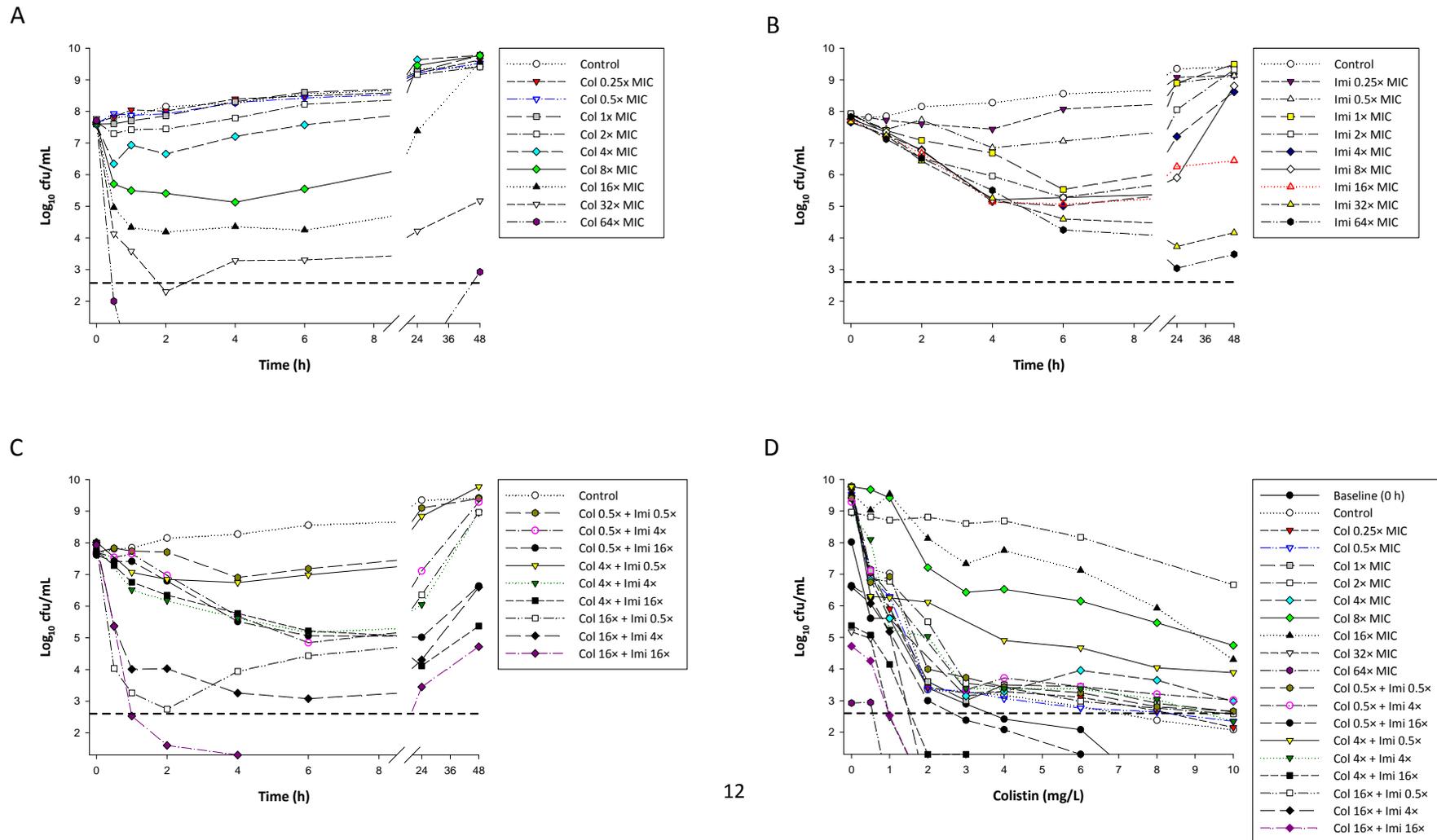


Figure S9: Time-kill curves for 19271 n/m at an inoculum of $\sim 10^6$ cfu/mL with (A) colistin and imipenem alone, and (B) in combination. Panel (C) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the limit of quantification (LOQ) is indicated by the horizontal broken line.

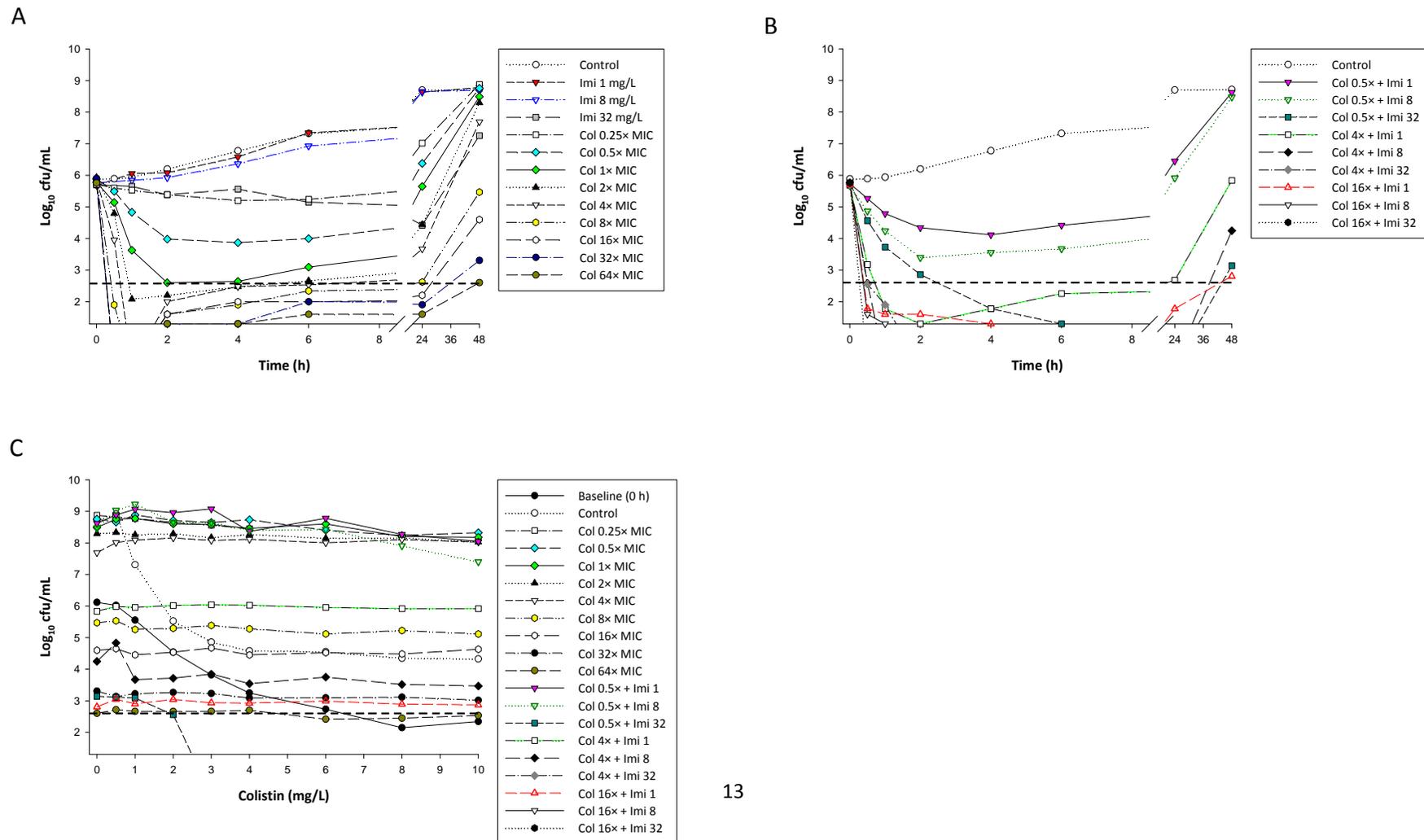


Figure S10: Time-kill curves for 19271 n/m at an inoculum of $\sim 10^8$ cfu/mL with (A) colistin and imipenem alone, and (B) in combination. Panel (C) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the limit of quantification (LOQ) is indicated by the horizontal broken line.

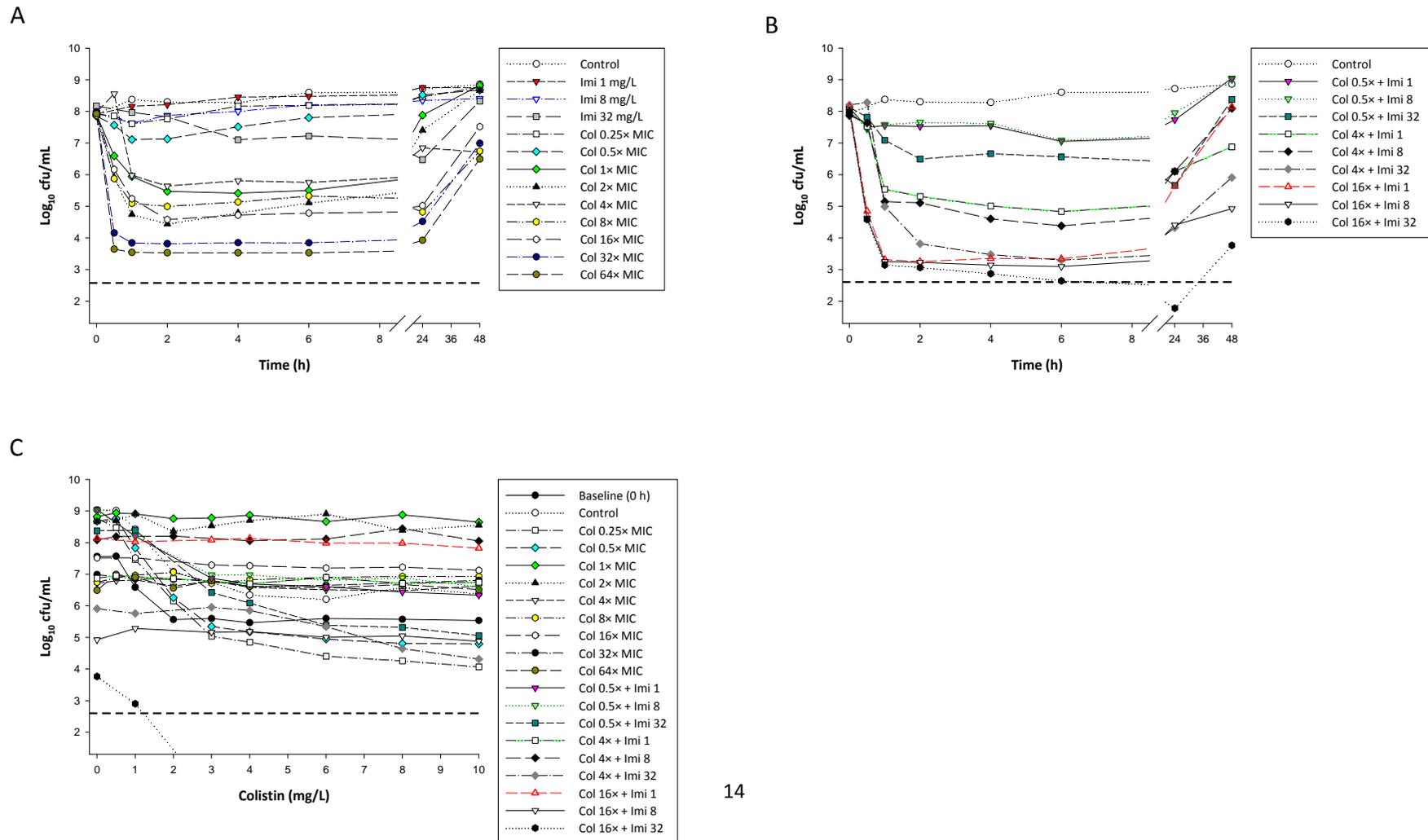
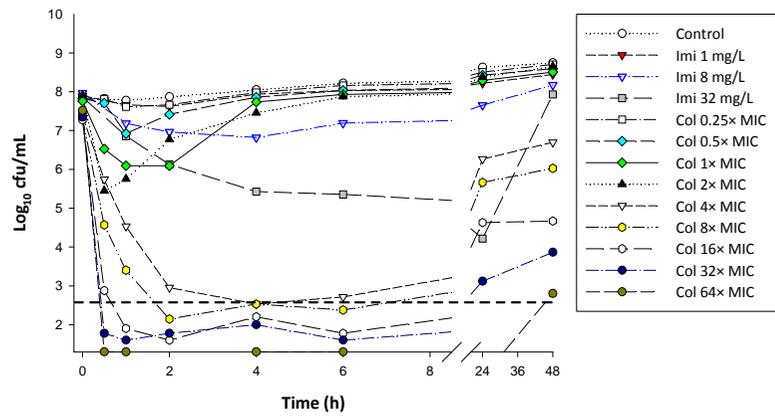
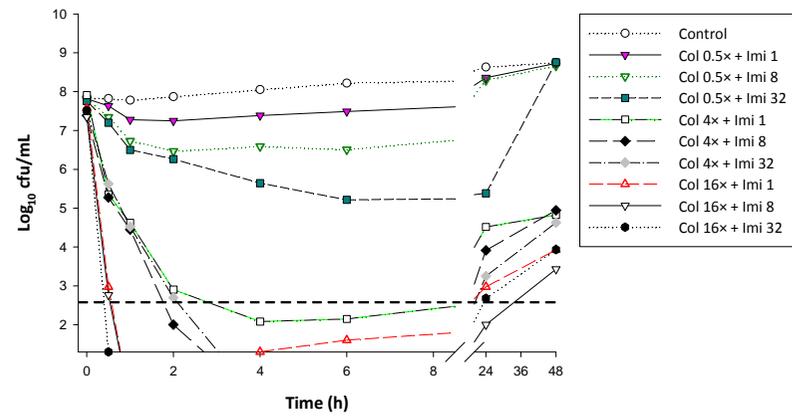


Figure S12: Time-kill curves for 20891 n/m at an inoculum of $\sim 10^8$ cfu/mL with (A) colistin and imipenem alone, and (B) in combination. Panel (C) shows the PAPs at baseline (0 h) and after 48 h exposure to colistin monotherapy, colistin/imipenem combination therapy, or neither antibiotic (control). The Y-axis starts from the limit of detection and the limit of quantification (LOQ) is indicated by the horizontal broken line.

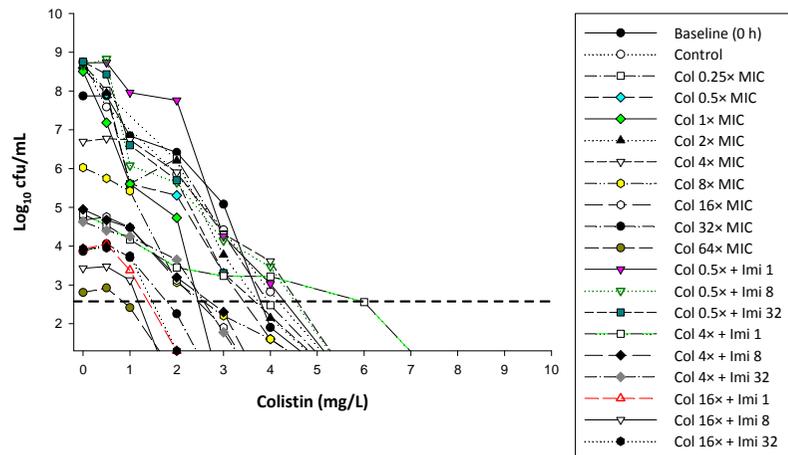
A



B



C



24 **Abstract**

25

26 Combination therapy may be required for MDR *Pseudomonas aeruginosa*. The aim of this
27 study was to systematically investigate bacterial killing and emergence of colistin resistance
28 with colistin and doripenem combinations against MDR *P. aeruginosa*. Studies were
29 conducted in a one-compartment *in vitro* PK/PD model for 96 h at two inocula ($\sim 10^6$ and
30 $\sim 10^8$ cfu/mL) against a colistin-heteroresistant reference strain (ATCC 27853) and colistin-
31 resistant-MDR clinical isolate (19147 n/m). Four combinations utilizing clinically achievable
32 concentrations were investigated. Microbiological response was examined by log changes
33 and population analysis profiles. Colistin (constant concentrations of 0.5 or 2 mg/L) plus
34 doripenem (peaks of 2.5 or 25 mg/L eight hourly, half-life 1.5 h) substantially increased
35 bacterial killing against both strains at the low inoculum, while combinations containing
36 colistin 2 mg/L increased activity against ATCC 27853 at the high inoculum; only colistin
37 0.5 mg/L plus doripenem 2.5 mg/L failed to improve activity against 19147 n/m at the high
38 inoculum. Combinations were additive or synergistic against ATCC 27853 in 16 and 11 of 20
39 cases (4 combinations across 5 sample points) at the 10^6 and 10^8 inocula, respectively; the
40 corresponding values for 19147 n/m were 16 and 9. Combinations containing doripenem 25
41 mg/L resulted in bacterial eradication of 19147 n/m at the low inoculum, and substantial
42 reductions in regrowth (including to below the limit of detection at ~ 50 h) at the high
43 inoculum. Emergence of colistin-resistant subpopulations in ATCC 27853 was substantially
44 reduced and delayed with combination therapy. This investigation provides important
45 information for optimization of colistin/doripenem combinations.

46

47

48 **Introduction**

49 Multidrug-resistant *Pseudomonas aeruginosa* is one of several important Gram-negative
50 bacteria emerging as significant pathogens worldwide (8, 50). With a very limited number of
51 therapeutic options remaining against these pathogens, and a lack of novel antimicrobial
52 agents in the drug development pipeline (31, 50), particularly those with activity against *P.*
53 *aeruginosa* (50), clinicians have been forced to re-examine the use of ‘old’, previously
54 discarded drugs such as the polymyxins (8, 41). Colistin (also known as polymyxin E) is a
55 multi-component cationic polypeptide antibiotic largely abandoned in the 1970s due to
56 concerns about the potential for nephro- and neuro-toxicity (16, 28). Colistin retains
57 significant *in vitro* activity against Gram-negative ‘superbugs’, and is often the only
58 therapeutic option available to treat infections caused by these pathogens (1, 28, 36). Several
59 institutions have already experienced outbreaks of multidrug-resistant (MDR) Gram-negative
60 bacteria resistant to all commercially available antibiotics except the polymyxins (6, 27, 35).
61 Of particular concern is that with the rapid increase in use of colistin over the last decade,
62 especially in critically-ill patients (8, 28), has come a concomitant increase in the number of
63 reports of resistance to colistin (1, 24, 28).

64

65 Having entered clinical use in 1959, colistin was never subjected to the scientific rigour
66 required of modern pharmaceuticals before they become available for use in patients. The
67 result has been a dearth of reliable pharmacokinetic (PK) and pharmacodynamic (PD)
68 information with which to guide therapy, and confusion has surrounded the optimal dosing
69 strategy. It is only very recently that crucial gaps in our knowledge of the PK and PD of
70 colistin have begun to be filled. Recent investigations into the PK of colistin in critically-ill
71 patients have revealed low and potentially sub-optimal plasma concentrations in a substantial
72 proportion of patients receiving currently recommended dosage regimens (17, 47). In

73 addition, both *in vitro* (3-4, 48, 52) and *in vivo* (23, 33) studies have shown the potential for
74 the rapid emergence of colistin resistance with monotherapy, with heteroresistance a likely
75 contributing factor; colistin heteroresistance has been identified in *Acinetobacter baumannii*
76 (29, 55), *Klebsiella pneumoniae* (48, 53), and most recently in *P. aeruginosa* (manuscript
77 submitted). The potential presence of colistin-resistant subpopulations prior to therapy in
78 heteroresistant strains, and the observation of rapid amplification of colistin-resistant
79 subpopulations with colistin monotherapy, suggests caution with the use of colistin
80 monotherapy and highlights the importance of investigating rational and novel colistin
81 combinations. The aim of the present study was to systematically investigate the extent of *in*
82 *vitro* bacterial killing and emergence of colistin resistance with colistin alone and in
83 combination with doripenem at both high and low inocula against *P. aeruginosa* using
84 clinically relevant dosage regimens. This was achieved by simulating, in an *in vitro* PK/PD
85 model, the PK of colistin formation and doripenem in humans over a range of clinically
86 achievable concentrations in critically-ill patients.

87

88 **Materials and Methods**

89 ***Bacterial isolates***

90 Two strains of *P. aeruginosa* were employed in this study: a colistin-heteroresistant reference
91 strain, ATCC 27853 (American Type Culture Collection, Rockville, MD, USA), and a non-
92 mucoid colistin-resistant multidrug-resistant (MDR) clinical isolate, 19147 n/m, obtained
93 from a patient with cystic fibrosis; the clinical isolate contained genes encoding IMP type
94 carbapenemase and CTX-M type extended-spectrum β -lactamase (ESBL). Heteroresistance
95 to colistin was defined as an isolate with a colistin minimum inhibitory concentration (MIC)
96 ≤ 2 mg/L in which subpopulations were able to grow in the presence of >2 mg/L colistin in
97 the population analysis profiles (PAPs; see below). MDR was defined as diminished

98 susceptibility to ≥ 2 of the following five drug classes: antipseudomonal cephalosporins,
99 antipseudomonal carbapenems, β -lactam- β -lactamase inhibitor combinations,
100 antipseudomonal fluoroquinolones, and aminoglycosides (45). MICs of colistin (sulphate)
101 and doripenem were each 1 mg/L for ATCC 27853, and 128 mg/L and 0.25 mg/L for 19147
102 n/m, respectively. MICs to colistin and doripenem for each isolate were determined in three
103 replicates on separate days in cation-adjusted Mueller-Hinton broth (CAMHB, Ca^{2+} at 23.0
104 $\mu\text{g/mL}$, Mg^{2+} at 12.2 $\mu\text{g/mL}$; Oxoid, Hampshire, England) via broth microdilution (13).
105 Resistance to colistin (13) and doripenem (15) was defined as MIC ≥ 4 mg/L. Strains were
106 stored in tryptone soy broth (Oxoid, Basingstoke, Hampshire, England) with 20% glycerol
107 (Ajax Finechem, Seven Hills, New South Wales, Australia) at -80°C in cryovials (Simport
108 Plastics, Boloel, Quebec, Canada).

109

110 ***Antibiotics and reagents***

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

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

124

125 ***Binding of doripenem in growth medium***

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

136

137 ***In vitro PK/PD model and colistin/doripenem dosing regimens***

138 Experiments to examine the microbiological response and emergence of resistance to various
139 dosage regimens of colistin and doripenem alone and in combination were conducted over 96
140 h at two different starting inocula ($\sim 10^6$ and $\sim 10^8$ cfu/mL) using a one-compartment *in vitro*
141 PK/PD model described previously (4) and below. Prior to each experiment, strains were
142 subcultured onto horse blood agar (Media Preparation Unit, The University of Melbourne,
143 Parkville, Australia) and incubated at 35°C for 24 h. One colony was then selected and grown
144 overnight in 10 mL of CAMHB, from which early log-phase growth was obtained. For a
145 starting inoculum of $\sim 10^6$ cfu/mL, a 1.0-mL aliquot of this early-log-phase bacterial
146 suspension was inoculated into each compartment at the commencement of the experiment to

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

151

152 The PK/PD model consisted of eight sealed containers (compartments) each containing 80
153 mL of CAMHB at 37°C and a magnetic stir bar to ensure adequate mixing. One compartment
154 acted as a control to define growth dynamics in the absence of antibiotic, while colistin
155 and/or doripenem were delivered into the remaining compartments to achieve the desired
156 constant concentration (colistin) or intermittent (doripenem) dosage regimens (see below). A
157 peristaltic pump (Masterflex L/S, Cole-Parmer, USA) was used to deliver sterile CAMHB
158 from separate central reservoirs into each compartment at a predetermined rate, displacing an
159 equal volume of CAMHB into a waste receptacle. Flow rates were calibrated prior to each
160 experiment and monitored throughout to ensure the system was performing optimally. For
161 colistin containing regimens, colistin was delivered as a constant concentration by spiking
162 colistin into the central reservoir prior to initiation of the experiment so that all media flowing
163 through the system (with the exception of the growth control compartment) contained a
164 constant concentration of colistin (Table 1); colistin was administered in this way to mimic
165 the flat plasma concentration-time profiles of formed colistin at steady-state observed in
166 critically-ill patients administered CMS (17, 47). For colistin-containing regimens at the
167 higher inoculum ($\sim 10^8$ cfu/mL), each compartment was initially filled with sterile drug-free
168 CAMHB to allow bacterial growth up to 10^8 cfu/mL in the absence of drug; subsequently, a
169 loading dose of colistin was administered to immediately attain the targeted colistin
170 concentration. For doripenem containing regimens, doripenem was injected into each
171 treatment compartment following bacterial inoculation to achieve the desired steady-state

172 peak concentration (C_{\max}), with intermittent 8-hourly dosing thereafter (Table 1); as
173 doripenem does not accumulate following multiple IV administration no loading dose was
174 required to achieve steady-state concentrations. The chosen flow rate simulated a doripenem
175 elimination half-life ($t_{1/2}$) of 1.5 h which approximates that in critically-ill patients (34).

176

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

185

186 *Microbiological response and the emergence of resistance to colistin*

187 Serial samples (0.6 mL) were collected aseptically at times shown in Table 1 from each
188 reservoir for viable cell counting and real-time PAPs, as well as determination of colistin and
189 doripenem concentrations. Viable counting and PAPs were conducted immediately after
190 sampling by spiral plating (WASP2 spiral plater, Don Whitley Scientific Ltd, UK) 50 μ L of
191 appropriately diluted sample (using 0.9% saline) onto either nutrient agar (viable counting) or
192 Mueller-Hinton agar (PAPs), followed by incubation at 35°C for 24 h (48 h for plates with
193 small colonies). Serial dilutions and plating with the spiral plater, which further dilutes the
194 sample, helped reduce the possibility of antibiotic carryover. PAPs plates were impregnated
195 with colistin (sulphate) at 0, 0.5, 1, 2, 3, 4, 5, 6, 8 and 10 mg/L; these concentrations were
196 chosen after consideration of the MICs and the colistin concentrations typically achievable in

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

203

204 ***Pharmacokinetic validation***

205 Samples (100 μ L) collected in duplicate from the *in vitro* PK/PD experiments were placed in
206 1.5 mL microcentrifuge tubes (Greiner Bio-One, Frickenhausen, Germany) and immediately
207 stored at -80°C until analysis; all samples were assayed within 4 weeks. Concentrations of
208 colistin were measured using high-performance liquid chromatography (HPLC) (26) with an
209 assay range for colistin sulfate of 0.10 to 6.00 mg/L. Doripenem concentrations were assayed
210 at ambient temperature using a validated reversed-phase HPLC method. The HPLC system
211 consisted of a Shimadzu LC-20AD Prominence liquid chromatograph, SIL-20AC HT
212 Prominence autosampler and SPD-M20A Prominence diode array detector (Shimadzu,
213 Columbia, MD, USA). To 100 μ L of sample, 100 μ L of 3-(N-morpholino)propanesulfonic
214 acid (MOPS) buffer and 400 μ L of methanol were added, vortexed and centrifuged at 10,000
215 rpm for 10 min. An aliquot of the sample (50 μ L) was injected onto a Phenosphere-NEXT 5
216 μ C18 column (250 mm \times 4.6 mm; Phenomenex, Torrance, California, USA). A gradient
217 elution procedure involving 100% methanol and 0.1% trifluoroacetic acid as the mobile
218 phases was used, the proportion of methanol increasing from 5% to 80% over 4 min then
219 returning to 5% over 0.5 min; the flow rate was 0.7 mL/min with detection at 311 nm. The
220 run time was 10 min. The assay range for doripenem was 0.5 to 32 mg/L; samples were
221 diluted when the expected doripenem concentrations were higher than the upper limit of

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

226

227 ***Pharmacodynamic analysis***

228 Microbiological response to monotherapy and combination therapy was examined using the
229 log change method comparing the change in \log_{10} (cfu/mL) from 0 h (CFU_0) to time t (6, 24,
230 48, 72 or 96 h; CFU_t) as shown:

$$231 \text{ Log change} = \log_{10}(CFU_t) - \log_{10}(CFU_0)$$

232 Single antibiotic or combination regimens causing a reduction of ≥ 1 - \log_{10} cfu/mL below the
233 initial inoculum at 6, 24, 48, 72 or 96 h were considered active. We considered synergy to be
234 a ≥ 2 - \log_{10} lower cfu/mL for the combination relative to its most active component at the
235 specified time (46); additivity was defined as a 1 to < 2 - \log_{10} lower cfu/mL for the
236 combination.

237

238 **Results**

239 ***Pharmacokinetic validation and doripenem binding***

240 The colistin drug concentrations achieved (mean \pm SD) were 0.45 ± 0.07 ($n = 22$), $1.76 \pm$
241 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
242 mg/L, respectively. Measured doripenem C_{\max} and trough concentration (C_{\min}) concentrations
243 were 51.47 ± 3.96 ($n = 30$) and 1.24 ± 0.42 ($n = 30$) mg/L for the targeted values of 50.0 and
244 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
245 25.0 and 0.62 mg/L. For the targeted doripenem C_{\max} of 2.5 mg/L, measured C_{\max}
246 concentrations were 2.45 ± 0.32 ($n = 50$), with all C_{\min} concentrations below the limit of

247 quantification (0.5 mg/L) of the HPLC assay. Typical simulated PK profiles for doripenem
248 dosage regimens of 25 and 50 mg/L 8-hourly are shown in Figure 1. The observed mean $t_{1/2}$
249 for the simulated intermittent doripenem dosage regimens was 1.55 ± 0.17 h ($n = 71$) for the
250 targeted value of 1.5 h; as C_{\min} for some dosage regimens was below the lower limit of
251 quantification of the HPLC assay, $t_{1/2}$ was not directly measured in all experiments. The f_u at
252 equilibrium was 0.95, indicating practical equivalence of total and unbound concentrations.

253

254 ***Microbiological response***

255 The initial inocula (mean \pm SD) were 6.20 ± 0.10 ($n = 11$) and 8.09 ± 0.08 ($n = 11$) \log_{10}
256 cfu/mL for ATCC 27853, and 6.30 ± 0.16 ($n = 9$) and 7.88 ± 0.28 ($n = 9$) \log_{10} cfu/mL for
257 19147 n/m, for the targets of 10^6 and 10^8 cfu/mL, respectively. The time-course profiles of
258 bacterial numbers achieved with all dosage regimens at both inocula are shown in Figure 2
259 (ATCC 27853) and Figure 3 (19147 n/m). Log changes of viable cell counts at each inoculum
260 with mono- and combination therapy are presented in Table 2.

261

262 *Colistin monotherapy.* Against ATCC 27853 at the 10^6 inoculum, colistin monotherapy
263 produced rapid and extensive initial killing at all concentrations, with colistin 2 and 5 mg/L
264 resulting in undetectable bacterial counts at 2 h (Fig. 2A). Substantial regrowth was evident at
265 6 h with colistin 0.5 mg/L and 24 h with colistin 2 mg/L, with regrowth approaching that of
266 the control by 24 h (0.5 mg/L) and 72 h (2 mg/L). No viable colonies were detected until 54 h
267 with colistin 5 mg/L, with subsequent regrowth to $\sim 4 \log_{10}$ cfu/mL observed at 96 h. An
268 inoculum effect with colistin monotherapy was observed, with substantially reduced initial
269 bacterial killing at the high compared to low inoculum with colistin 0.5 and 2 mg/L (Fig. 2D).
270 While rapid and extensive initial bacterial killing to below the limit of detection remained at
271 the high inoculum with colistin 5 mg/L, substantial regrowth (to $\sim 3.5 \log_{10}$ cfu/mL) had

272 occurred by 6 h, with regrowth to above the level of the initial inoculum by 30 h. Against the
273 colistin-resistant isolate, bacterial growth in the presence of colistin 5 mg/L was essentially
274 no different to that of the growth control at either inoculum (Fig. 3A and C).

275

276 *Doripenem monotherapy.* Against ATCC 27853 at the 10^6 inoculum, all doripenem regimens
277 (2.5, 25 or 50 mg/L, 8-hourly) produced initial bacterial killing of $\sim 2.5\text{-log}_{10}$ cfu/mL, with
278 regrowth beginning by 6 h (Fig. 2B). Regrowth close to control levels had occurred by 48,
279 72, and 96 h with concentrations of 2.5, 25 and 50 mg/L, respectively. At the high inoculum
280 all doripenem concentrations produced a similar killing profile with the 2.5 mg/L 8-hourly
281 regimen resulting in bacterial counts consistently $\sim 0.5\text{-}$ to 1-log below control values, and 25
282 and 50 mg/L regimens bacterial counts $\sim 1.5\text{-}$ to 3-log below control values (Fig. 2E). Against
283 the MDR isolate, doripenem 2.5 mg/L 8-hourly produced only minimal bacterial killing ($\sim 1\text{-}$
284 to 2-log_{10} kill) at each inoculum, with regrowth close to control values by 24 to 48 h (Fig. 3A
285 and C). Higher doripenem concentrations (25 and 50 mg/L) produced rapid initial killing of
286 $\sim 3\text{-log}$ at 6 h, with subsequent regrowth to within $\sim 1\text{-log}$ of control values at 96 h (Fig. 3A
287 and C). No inoculum effect was observed with doripenem against either strain.

288

289 *Combination therapy.* Against ATCC 27853, the addition of doripenem 2.5 or 25 mg/L to
290 colistin 0.5 mg/L produced an initial (i.e., up to 8 h) additional bacterial kill of $\sim 2.5\text{-log}_{10}$
291 cfu/mL compared with the most active monotherapy (colistin) at the low inoculum, and
292 resulted in undetectable bacterial counts no later than 3 h (Table 2). Both combinations
293 resulted in synergy or additivity at most time points across 96 h (Table 2). Synergy was
294 particularly evident with the combination of colistin 0.5 mg/L and doripenem 2.5 mg/L, with
295 $\sim 3\text{-}$ to 4-log_{10} greater kill at most time points. Nevertheless, by 96 h regrowth with this
296 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 $\sim 3.5 \log_{10}$ cfu/mL kill over the most active monotherapy achieved at 8h with
304 doripenem 2.5 mg/L, and an additional $\sim 5 \log_{10}$ 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

322 2). Substantially greater killing was observed with combinations containing doripenem 25
323 mg/L. The addition of doripenem 25 mg/L to colistin (0.5 or 2 mg/L) produced substantial
324 reductions in \log_{10} cfu/mL over that of equivalent doripenem monotherapy by 8 h (with
325 colistin 2 mg/L) and 29 h (with colistin 0.5 mg/L) (Fig. 3D). No viable bacteria were detected
326 at ~50 h with both combinations, with regrowth at 96 h substantially below (by $\sim 3.5 - 5 \log_{10}$
327 cfu/mL) that of equivalent doripenem monotherapy (Fig. 3D).

328

329 *Emergence of colistin resistance*

330 Apart from a small shift to the right from 0 to 96 h at the 10^6 cfu/mL inoculum, the PAPs for
331 ATCC 27853 at 96 h closely matched those observed at baseline at both inocula. With this
332 strain, a small number of colistin-resistant colonies were detected at baseline at the high
333 inoculum, and for both inocula following 96 h incubation in the model (Table 3). Colistin 0.5
334 or 2 mg/L resulted in substantial increases in the proportion of colistin-resistant
335 subpopulations at both inocula (Fig. 4 and Table 3). With colistin 5 mg/L, the substantially
336 lower growth at 96 h ($\sim 4.3 \log_{10}$ cfu/mL) using an initial inoculum of 10^6 makes comparison
337 of the PAPs at this time difficult. However, at the 10^8 inoculum a substantial increase in
338 colistin-resistant subpopulations was evident by 24 h with colistin 5 mg/L monotherapy (Fig.
339 3 and Table 3). For 19147 n/m, the PAPS at baseline and across the 96 h incubation period
340 did not change irrespective of inoculum or colistin treatment (data not shown).

341

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

347 (Fig. 4). For example, with a starting inoculum of 10^8 cfu/mL, the combination of colistin 2
348 mg/L plus doripenem 2.5 mg/L resulted in substantially fewer colonies growing in the
349 presence of ≥ 4 mg/L colistin at 96 h compared with equivalent colistin monotherapy (Fig.
350 4F). The number of resistant colonies was reduced even further with the combination of
351 colistin 0.5 mg/L plus doripenem 25 mg/L, despite a similar level of growth at this time with
352 all three regimens. Combination therapy had no effect on colistin resistance of the MDR-
353 colistin-resistant isolate (data not shown).

354

355 Discussion

356 Colistin is increasingly used as salvage therapy in critically-ill patients for otherwise
357 untreatable MDR infections (16, 28). However, regrowth of colistin-susceptible *P.*
358 *aeruginosa* with colistin (or polymyxin B) monotherapy is commonly observed (4, 10, 19,
359 23, 51), even with colistin concentrations well above those which can be safely achieved
360 clinically. In addition, recent population PK studies employing currently recommended CMS
361 dosage regimens indicate that the plasma colistin concentrations achieved in critically-ill
362 patients are in many cases suboptimal (17, 47). Given the potential for the rapid emergence of
363 colistin resistance with monotherapy, combination therapy against *P. aeruginosa* has been
364 suggested as a possible means by which to increase antimicrobial activity and reduce the
365 development of resistance (30). We systematically investigated the effectiveness of colistin
366 alone and in combination with doripenem against a colistin heteroresistant strain and a MDR-
367 colistin-resistant isolate of *P. aeruginosa*. Doripenem was chosen because of its high potency
368 against MDR *P. aeruginosa* (11, 39) and its low potential for selection of carbapenem-
369 resistant *P. aeruginosa* (20, 38, 49). As some data show that activity of colistin (10) and
370 carbapenems alone (37) is attenuated at high compared to low inocula, in the present study

371 experiments were conducted at both $\sim 10^6$ and $\sim 10^8$ cfu/mL; the latter inoculum mimics the
372 high bacterial densities found in some infections.

373

374 The dosage regimens of colistin and doripenem used in the present study were carefully
375 chosen to reflect the plasma concentration-time profiles achieved in critically-ill patients.
376 Intravenous administration of CMS, the parenteral formulation of colistin, results in average
377 steady-state plasma colistin concentrations of $\sim 2 - 3$ mg/L, with some patients achieving
378 concentrations up to ~ 10 mg/L (17, 32, 47). As colistin concentrations at steady-state remain
379 more or less constant (17, 47), colistin was administered as a constant infusion. We have
380 previously demonstrated that colistin is almost entirely unbound in CAMHB (3). Thus,
381 colistin concentrations of 0.5 and 2 mg/L used in our study are clinically achievable,
382 assuming plasma binding of colistin in patients is similar to that in animals (i.e. $\sim 50\%$ bound)
383 (26). Unfortunately, although the knowledge of total plasma colistin concentrations achieved
384 in patients is increasing, there is currently no information on unbound plasma concentrations
385 in humans. Though the majority of PK data on doripenem has been obtained in healthy
386 volunteers, plasma concentration-versus-time profiles in patients appear similar to those in
387 healthy volunteers (40). Doripenem is typically administered intermittently every 8 h, with a
388 standard 500 mg dose achieving a C_{\max} of ~ 25 mg/L (7, 15). As binding of doripenem in the
389 growth media was minimal, all doripenem concentrations employed in the combinations are
390 readily achieved in plasma after consideration of protein binding (7, 14-15, 21).

391

392 To our knowledge, this is the first study to investigate the combination of colistin plus
393 doripenem against *P. aeruginosa* using an *in vitro* PD model and to utilise colistin PK data
394 recently obtained from critically-ill patients (discussed subsequently). An inoculum effect
395 was generally observed for colistin monotherapy, whereas no obvious inoculum effect was

396 present for doripenem (Figs.2 and 3). The addition of doripenem to colistin resulted in
397 substantial improvements in bacterial killing over equivalent monotherapy against the MDR-
398 colistin-resistant isolate at both inocula, particularly with a doripenem concentration of 25
399 mg/L. Though the benefits in overall antibacterial activity with the combination were slightly
400 less pronounced against the colistin-susceptible but -heteroresistant strain, combination
401 regimens nevertheless resulted in substantial improvements in bacterial killing, particularly
402 with combinations containing colistin 2 mg/L. Overall, our data suggests that the addition of
403 doripenem to even low concentrations of colistin (e.g., 0.5 mg/L) can substantially improve
404 antibacterial activity. Given the current last-line status of colistin therapy, we reported not
405 only synergy but also additivity as even a relatively small increase in activity with clinically
406 achievable concentrations of both antibiotics may be beneficial to patient care.

407

408 Previous studies employing static time-kill methods have examined colistin in combination
409 with a carbapenem (imipenem, meropenem, or doripenem) against *P. aeruginosa*, with mixed
410 results (2, 12, 43-44, manuscript submitted). In these previous reports, investigations were
411 undertaken for no longer than 48 h (usually 24 h) with a single dose of each antibiotic
412 administered at the commencement of treatment. Of these studies, only our previous study
413 employed multiple inocula and investigated the emergence of colistin resistance (manuscript
414 submitted); that study included both isolates used in the present study. While concentrations
415 of antibiotics between that and the present study are not directly comparable, and the former
416 study examined colistin in combination with imipenem, the activity of colistin combined with
417 either imipenem or doripenem was similar across 48 h (the duration of the former study) at
418 both inocula against ATCC 27853. However, substantial differences were evident against the
419 MDR-colistin-resistant isolate. In the static model, combinations with concentrations as high
420 as 32 mg/L colistin plus 16× MIC imipenem failed to reduce bacterial numbers to below the

421 limit of detection at any time. In stark contrast, bacterial eradication was achieved in the
422 PK/PD model with combinations containing colistin (0.5 or 2 mg/L) and doripenem 25 mg/L
423 no later than 24 h at the low inoculum, and bacteria reduced to below detectable levels at
424 approximately 48 h with the same combinations at the high inoculum. This highlights the
425 importance of simulating PK profiles when examining PD responses.

426

427 Though *P. aeruginosa* can undergo adaptive resistance to polymyxins (18), the report of
428 colistin heteroresistance in *P. aeruginosa* (manuscript submitted), and changes in PAPs
429 following treatment with colistin monotherapy (4, 10, manuscript submitted), suggest
430 amplification of pre-existing colistin-resistant subpopulations is a contributing factor to the
431 regrowth observed with colistin monotherapy. This was similarly observed in the present
432 study with colistin monotherapy. Though the meaningful interpretation of PAPs is difficult
433 where combination therapy has led to extensive killing, an important finding of the present
434 study is that when bacterial numbers were comparable (within $\sim 1\text{-}2 \log_{10}$ cfu/mL of
435 equivalent monotherapy) combination therapy against the colistin heteroresistant strain at
436 both inocula substantially reduced and delayed the emergence of colistin-resistant
437 subpopulations. Whereas colistin-resistant colonies emerged rapidly (often within 24 h) with
438 colistin monotherapy, with combination therapy resistant colonies generally emerged later
439 (following 72 to 96 h of treatment) and formed a substantially smaller proportion of the
440 overall bacterial population (Table 3). In addition, the most resistant subpopulations (i.e.,
441 those growing in the presence of colistin 10 mg/L on the PAPs plates) were absent with
442 combination therapy. In contrast, we previously reported changes in the PAPs with colistin
443 and imipenem combination therapy in a static time-kill model generally mirrored those
444 observed with equivalent exposure to colistin monotherapy (manuscript submitted). Loss of
445 imipenem due to degradation in the static experiments likely contributed to this result (22).

446 Intermittent dosing of doripenem in the present study replenishes doripenem concentrations
447 and avoids the combination effectively becoming colistin monotherapy over time. This
448 reported difference highlights once again the importance of PK/PD models in assessing
449 activity and emergence of resistance of antimicrobial therapy.

450

451 We have previously suggested two possible reasons for an enhanced PD effect observed with
452 the combination of colistin and a carbapenem (9). Subpopulation synergy involves one drug
453 killing the resistant subpopulation(s) of the other drug, and *vice versa*. ATCC 27853 is
454 colistin-heteroresistant, indicating the existence of colistin-resistant subpopulations prior to
455 therapy. Though regrowth occurred with this strain with all combinations, it was considerably
456 reduced with combinations containing each drug at the higher concentration, particularly over
457 the first 48 to 72 h. Interestingly, high-level colistin resistance did not emerge despite the
458 regrowth. While subpopulation synergy may have contributed to an enhanced PD effect
459 against this isolate, it cannot explain the substantially enhanced activity of colistin/doripenem
460 combinations against the MDR-colistin-resistant isolate given its near complete resistance to
461 colistin (MIC 128 mg/L). This enhanced activity occurred despite the presence of enzymes
462 active against carbapenems. Mechanistic synergy involves colistin and doripenem acting on
463 different cellular pathways to increase the rate or extent of killing of the other drug. It is
464 possible permeabilization of the outer membrane by colistin (56) resulted in substantially
465 increased concentrations of doripenem in the periplasm, allowing greater access to the critical
466 penicillin-binding proteins located on the cytoplasmic membrane where the carbapenems act
467 (42, 54). Subpopulation and mechanistic synergy are not mutually exclusive, and both may
468 operate simultaneously. Further investigations are ongoing to elucidate the mechanism(s)
469 underpinning the enhanced PD activity observed.

470

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

480

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488

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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

682

	Dosage regimens at $\sim 10^6$ and $\sim 10^8$ 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.

684 **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.*
 685 *aeruginosa*. Gray background indicates activity (a reduction of ≥ 1 -log₁₀ cfu/mL below the initial inoculum); green background indicates synergy
 686 (a ≥ 2 -log₁₀ decrease in the number of cfu/mL between the combination and its most active component); red background indicates additivity (a
 687 1.0 to < 2 -log₁₀ 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	
	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	
	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
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	
10^8		6	-	-	-0.99	-0.01	-1.35	-1.57	-0.69	-6.46	-0.27	-4.24	

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^{††} 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

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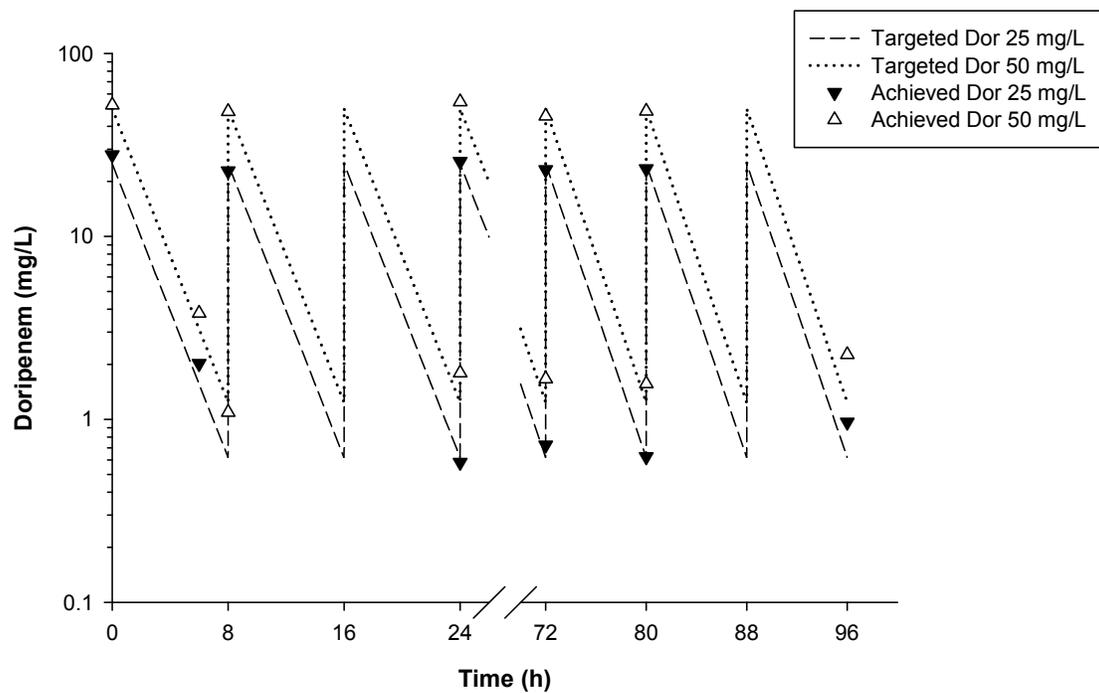
690 **Table 3:** Proportion of colistin-resistant subpopulations in *P. aeruginosa* ATCC 27853 at various times in the *in vitro* PK/PD model

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

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^{§§} ND: No colistin-resistant subpopulations detected.

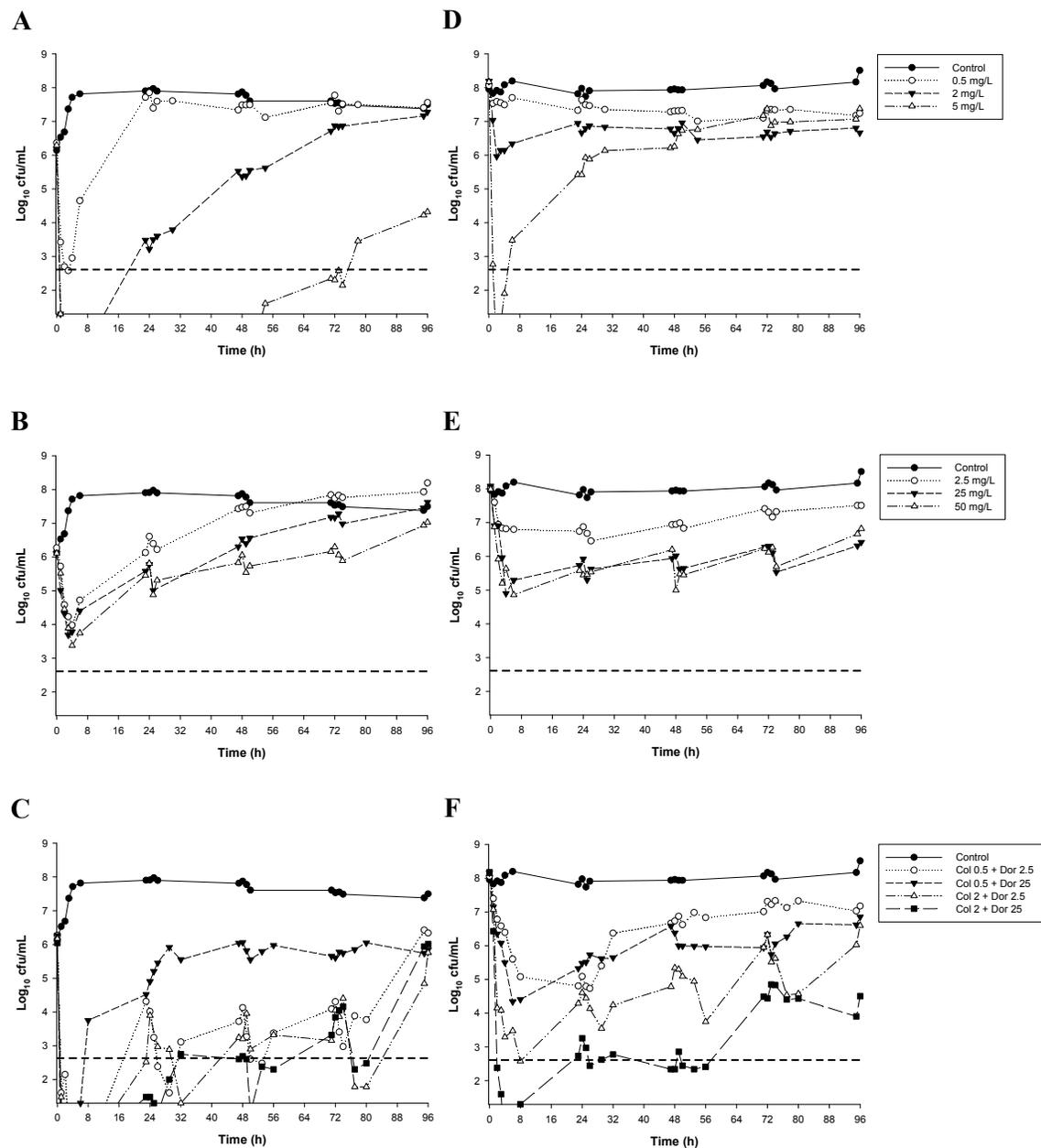


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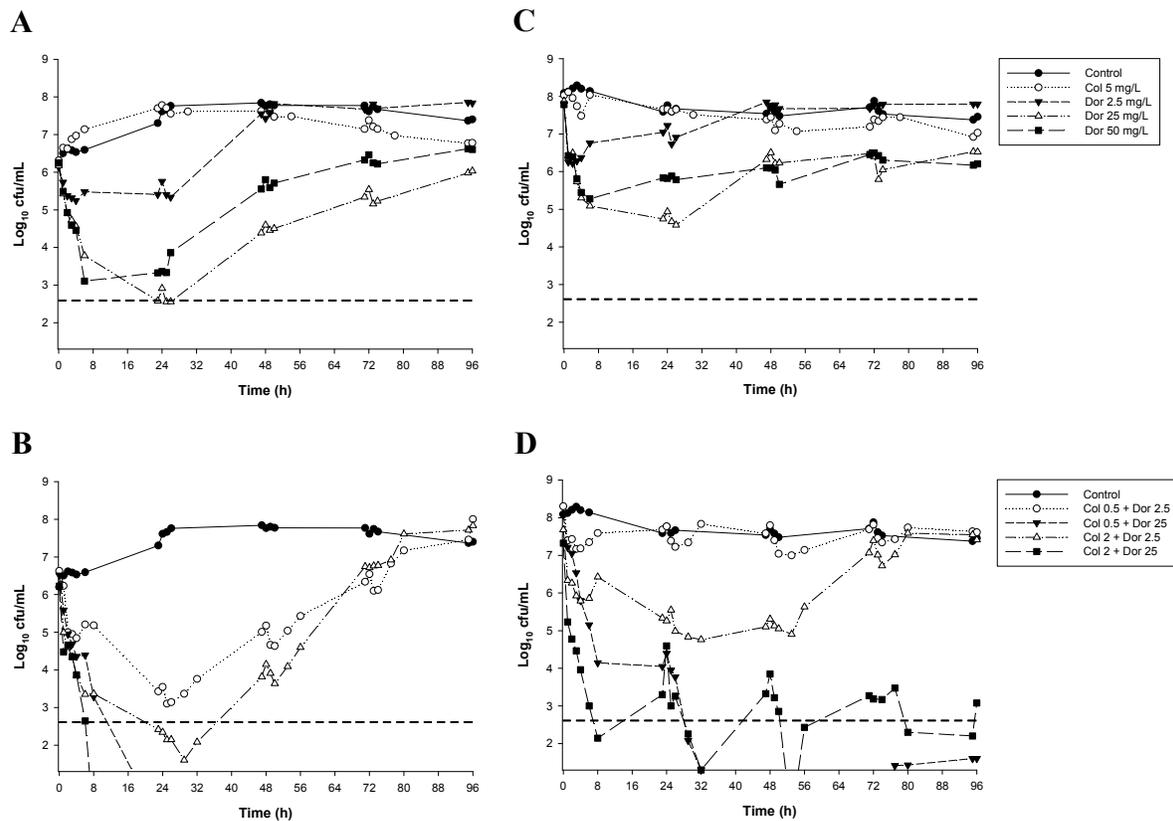
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696 **Figure 1.** Targeted doripenem (Dor) pharmacokinetic profiles for 25 and 50 mg/L 8-hourly regimens with
697 measured Dor concentrations.



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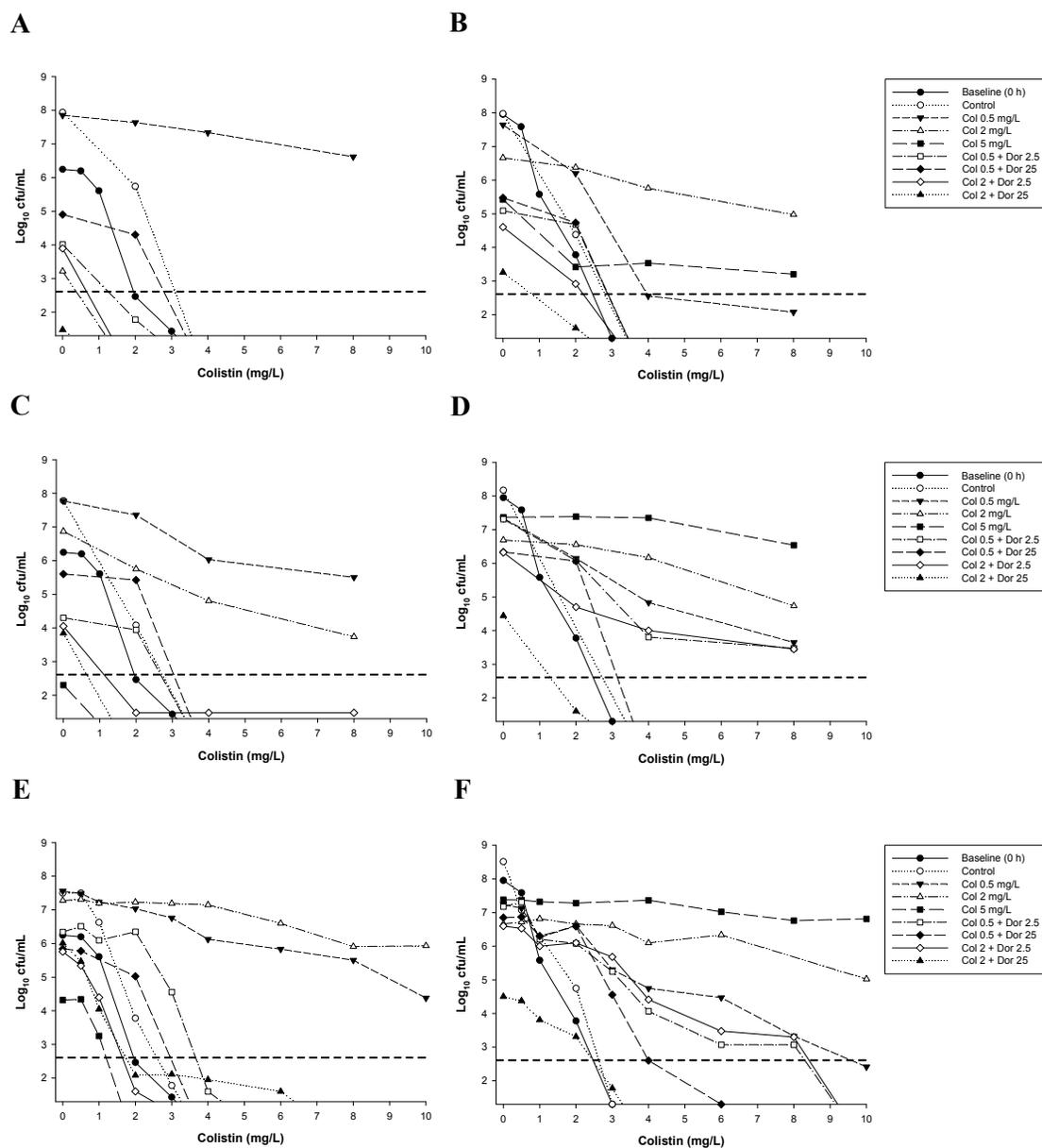
699 **Figure 2.** Time-kill curves for colistin monotherapy (Panels A and D), doripenem
 700 monotherapy (Panels B and E) and the combination (Panels C and F) against ATCC 27853 at
 701 the 10^6 cfu/mL (left-hand panels) and 10^8 cfu/mL (right-hand panels) inocula. The y-axis
 702 starts from the limit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



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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.

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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.

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