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Optimising antibiotic administration against difficult-to-treat infections caused by the Gram-negative 'superbug' *Pseudomonas aeruginosa*

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B. Pharm., M. Phil.

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Dedicated to

Eshaal, Abiha

and Ali

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Abstract

The rapid increase of antibiotic resistance globally presents one of the greatest threats to human health. This situation is exacerbated by a shortage of new antibiotics, coupled with the suboptimal use of antibiotics. The Gram-negative 'superbug' *Pseudomonas aeruginosa* has a large armamentarium of resistance mechanisms and causes difficult-to-treat infections in critically ill patients and patients with cystic fibrosis (CF). In light of the current increase in multidrug-resistance (MDR), there is an urgent need for new antibiotics. Unfortunately, the development process for new antibiotics takes many years. Therefore, one of the current challenges is to suppress further resistance emergence through use of optimised therapy with currently available antibiotics.

Significant activity of fosfomycin, an antibiotic developed decades ago, has been well documented in the literature against *P. aeruginosa*, but this antibiotic has remained underutilised due to a scarcity of information on its pharmacokinetics (PK), pharmacodynamics (PD) and the relationship between the two (*i.e.* PK/PD). Thus, there is an urgent need to investigate exposure-response relationships of this 'old' antibiotic. This thesis describes the first such investigation using an extensive dose-ranging and dose-fractionation design. The study was conducted in a dynamic *in vitro* infection model to identify the most predictive PK/PD index driving fosfomycin activity against *P. aeruginosa*. The study provides important information to guide the rational clinical use of fosfomycin.

For people with CF, respiratory infections are a main driver of early death. Acute infective exacerbations (AIEs) of chronic *P. aeruginosa* infections are associated with declining lung function and presence of biofilm and hypermutable phenotype makes them difficult to treat. Such infections often require repetitive cycles of antibiotics, and each suboptimal treatment leads to resistance development. Only a few studies have assessed antibiotic therapies against hypermutable *P. aeruginosa*, either against planktonic bacteria or against static-phase biofilm bacteria. Most studies have found emergence of resistance following the use of a single antibiotic, indicating that monotherapy is not a tangible option for treatment of AIEs. Thus, empirical combination regimens of available antibiotics are used clinically, but information about the effectiveness of different modes and routes of administration of combination regimens against planktonic and biofilm bacteria of hypermutable *P. aeruginosa* strains under dynamic conditions is scarce in the literature.

This project applied modern principles of antimicrobial PK and PD to currently available antibiotics to generate essential information required for the rational design of dosing strategies that maximise bacterial killing and suppress the emergence of resistance. The Center for Disease Control (CDC) biofilm reactor (CBR), which is a dynamic *in vitro* infection model, was utilised to investigate three different two-drug combinations of antibiotics against planktonic

and biofilm bacteria of hypermutable *P. aeruginosa* strains. Across the three studies, the impact of the mode and route of delivery of the antibiotics on the time-course of bacterial killing and emergence of resistance was investigated by simulating the clinically relevant concentrations of the antibiotics. The research described in this thesis provides new information to clinicians regarding important factors to consider when prescribing antibiotic regimens for patients with difficult-to-treat infections caused by *P. aeruginosa*.

Thesis including published works declaration

Statement of Authorship

Declaration for thesis based or partially based on conjointly published or unpublished work

In accordance with Monash University Doctorate Regulation 17.2 Doctor of Philosophy and Research Master's regulations 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 and that, 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 **3** original research papers published in peer reviewed journals and one original manuscript is ready for submission (under internal review with co-authors) to a peer-reviewed journal for publication. The core theme of the thesis is **Optimising antibiotic administration against difficult-to-treat infections caused by the Gram-negative 'superbug' *Pseudomonas aeruginosa***. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the **Centre for Medicine Use and Safety, Monash Institute of Pharmaceutical Sciences** under the supervision of **Associate Professor Cornelia B. Landersdorfer**.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research. **Chapters 2, 3, and 4** contain the manuscripts published and have not been modified for inclusion in this thesis.

A summary of my contribution to the work involved is tabulated below:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
2	Elucidation of the pharmacokinetic / pharmacodynamic determinants of fosfomycin activity against *Pseudomonas aeruginosa* using a dynamic *in vitro* model	Published *JAC* 2018; 73:1570-1578	Primary authorship, experimental design, preparation and execution of laboratory experiments, data analysis, interpretation, fitting, formulation of the conclusions and hypothesis arising from the results, writing first draft and subsequent revisions of the manuscript. (**80%)**	1) Anton Y Peleg, Supplied clinical isolates and editorial review (2.5%) 2) Michelle P McIntosh, Editorial review (2.5%) 3) Ian K Styles, LC-MS/MS assistance (2.5%) 4) Elizabeth B Hirsch, Editorial review (2.5%), 5) Cornelia B Landersdorfer, Supervision and editorial review (2.5%) 6) Phillip J Bergen, Supervision, senior/corresponding authorship, experimental design and editorial review (7.5%)	*No* *No* *Yes* *No* *No* *No*
3	Synergistic meropenem-tobramycin combination dosage regimens against clinical hypermutable *Pseudomonas aeruginosa* at simulated epithelial lining fluid concentrations in a dynamic biofilm model	Published *AAC* 2019; 63: e01293-19	Primary authorship, optimisation and aseptic functioning of CBR model, experimental design, preparation and execution of laboratory experiments, data analysis, interpretation of results, formulation of the conclusions and hypothesis arising from the results, writing first draft and subsequent revisions of the manuscript. **(75%)**	1) Phillip J Bergen, Supervision and editorial review (2.5%) 2) Tae Hwan Kim, Editorial review (2.5%) 3) Seung Eun Chung, LC-MS/MS assistance (2.5%) 4) Anton Y Peleg, Supplied clinical isolates and editorial review (2.5%) 5) Antonio Oliver, Supplied reference isolate and editorial review (2.5%) 6) Roger L Nation, Intellectual input and editorial review (2.5%) 7) Cornelia B Landersdorfer, Supervision, senior/corresponding authorship, experimental design and editorial review (10%)	*No* *No* *No* *No* *No* *No* *No*
4	Clinically relevant epithelial lining fluid concentrations of meropenem with ciprofloxacin provide synergistic killing and resistance suppression of hypermutable *Pseudomonas aeruginosa* in a dynamic biofilm model	Published *AAC* 2020; 64: e00469-20	Primary authorship, experimental design, preparation and execution of laboratory experiments, data analysis, interpretation of results, formulation of the conclusions and hypothesis arising from the results, writing first draft and subsequent revisions of the manuscript. **(70%)**	1) Phillip J Bergen, Supervision and editorial review (2.5%) 2) Jessica R Tait, Experimental assistance (5%) 3) Steven Wallis, LC-MS/MS assistance (2.5%) 4) Anton Y Peleg, editorial review (2.5%) 5) Jason A Roberts, Editorial review (2.5%) 6) Antonio Oliver, Supplied reference isolate and editorial review (2.5%) 7) Roger L Nation, Intellectual input and editorial review (2.5%) 8) Cornelia B Landersdorfer, Supervision, senior/corresponding authorship, experimental design and editorial review (10%)	*No* *Yes* *No* *No* *No* *No* *No* *No*

I have not renumbered sections of accepted or published papers in order to generate a consistent presentation within the thesis.

Candidate's Signature		**Date** 27/07/2020

Print name: (Hajira Bilal)

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author, I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor's Signature		**Date** 27/07/2020

Print name: (Cornelia Barbara Landersdorfer)

General declaration

Chapter 5 contain unpublished work and this is presented similarly to accepted or submitted Chapters. This manuscript will be submitted soon to a peer-reviewed journal for publication.

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution*	Co-author(s), Monash student Y/N*
5	Synergistic ceftazidime and tobramycin combinations for clinical hypermutable *Pseudomonas aeruginosa* isolates; an innovative dosing approach to enhance bacterial killing and mitigate resistance in a dynamic biofilm model	manuscript being prepared for journal submission	Primary authorship, experimental design, preparation and execution of laboratory experiments, data analysis, interpretation of results, formulation of the conclusions, hypothesis arising from the results, writing first draft and subsequent revisions of the manuscript. **(75%)**	1) Phillip J Bergen, Supervision and editorial review (2.5%) 2) Wee L Lee, LC-MS/MS assistance (2.5%) 3) Anton Y Peleg, editorial review (2.5%) 4) Antonio Oliver, Supplied reference isolate and editorial review (2.5%) 5) Roger L Nation, Intellectual input and editorial review (5%) 6) Cornelia B Landersdorfer, Supervision, senior/corresponding authorship, experimental design and editorial review (10%)	*No* *No* *No* *No* *No* *No*

Candidate's Signature		**Date** 27/07/2020

Print name: (Hajira Bilal)

Main Supervisor's Signature		**Date** 27/07/2020

Print name: (Cornelia Barbara Landersdorfer)

Publications during enrolment

1. Bilal H, Peleg AY, McIntosh MP, Styles IK, Hirsch EB, Landersdorfer CB and Bergen PJ. Elucidation of the pharmacokinetic/pharmacodynamic determinants of fosfomycin activity against *Pseudomonas aeruginosa* using a dynamic *in vitro* model. ***J Antimicrob Chemother***., 2018; 73: 1570-1578.

2. Bilal H, Bergen PJ, Kim TH, Chung SE, Peleg AY, Oliver A, Nation RL, Landersdorfer CB. Synergistic meropenem-tobramycin combination dosage regimens against clinical hypermutable *Pseudomonas aeruginosa* at simulated epithelial lining fluid concentrations in a dynamic biofilm model. ***Antimicrob Agents Chemother***., 2019; 63(11): e01293-19.

3. Bilal H, Bergen PJ, Tait JR, W SC, Peleg AY, Roberts JA, Oliver A, Nation RL, Landersdorfer CB. Clinically relevant epithelial lining fluid concentrations of meropenem with ciprofloxacin provide synergistic killing and resistance suppression of hypermutable *Pseudomonas aeruginosa* in a dynamic biofilm model. ***Antimicrob Agents Chemother***., 2020; 64(7): e00469-20.

4. Huma ZE, Javed I, Zhang Z, Bilal H, Sun Y, Hussain SZ, Davis TP, Otzen DE, Landersdorfer CB, Ding F, Hussain I, Ke PC. Nanosilver mitigates biofilm formation via FapC Amyloidosis inhibition. ***Small***, 2020: 16(21): e1906674-84.

5. Tait JR, Bilal H, Kim TH, Oh A, Peleg AP, Boyce J, Bergen PJ, Oliver A, Nation RL, Landersdorfer CB. Pharmacodynamics of ceftazidime plus tobramycin combination dosage regimens against hypermutable *Pseudomonas aeruginosa* isolates at simulated epithelial lining fluid concentrations in a dynamic *in vitro* infection model. ***Antimicrob Agents Chemother.***, 2020 (submitted)

6. Tait JR, Bergen PJ, Bilal H, Roberts JA, Nation RL, Landersdorfer CB. Optimisation of piperacillin-tobramycin combination dosage regimens against *Pseudomonas aeruginosa* isolates via mechanism-based modelling. (in progress)

Conference presentations during enrolment

1. Bilal H, Peleg AY, McIntosh MP, Styles IK, Hirsch EB, Landersdorfer CB and Bergen PJ. Elucidation of the pharmacokinetic/pharmacodynamic determinants of fosfomycin activity against *Pseudomonas aeruginosa* using a dynamic *in vitro* model, Poster presentation at the PharmAlliance Conference, Melbourne, Australia, 6th Feb, 2019.

2. Bilal H, Bergen PJ, Kim TH, Chung SE, Peleg AY, Oliver A, Nation RL, Landersdorfer CB. Synergistic meropenem-tobramycin combination dosage regimens against clinical hypermutable *Pseudomonas aeruginosa* at simulated epithelial lining fluid concentrations in a dynamic biofilm model, Poster presentation at the 29th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) conference, Amsterdam, Netherlands, 13-16th April, 2019.

3. Bilal H, Bergen PJ, Kim TH, Chung SE, Peleg AY, Oliver A, Nation RL, Landersdorfer CB. Pharmacodynamics of meropenem-tobramycin combination dosage regimens against clinical hypermutable *Pseudomonas aeruginosa* at simulated epithelial lining fluid concentrations in a dynamic biofilm model, Oral presentation at the International Society for Anti-Infective Pharmacology (ISAP) conference, Rotterdam, Netherlands, 14th April, 2019.

4. Bilal H, Optimising antibiotic dosing against biofilm infections, Oral talk at 3 Minute Thesis (3MT) competition, Monash University, 21st June, 2019 (Among Faculty finalists).

5. Bilal H, Pharmacodynamics of meropenem-tobramycin combination dosage regimens against clinical hypermutable *Pseudomonas aeruginosa* at simulated epithelial lining fluid concentrations in a dynamic biofilm model, Poster at 14th Annual Postgraduate Research Symposium, MIPS, Monash University, 26th July, 2019.

6. Bilal H, Bergen PJ, Kim TH, Chung SE, Peleg AY, Oliver A, Nation RL, Landersdorfer CB. Pharmacodynamics of meropenem-tobramycin combination dosage regimens against clinical hypermutable *Pseudomonas aeruginosa* at simulated epithelial lining fluid concentrations in a dynamic biofilm model", Poster at Her Research Matters Symposium, MIPS, Monash University, Melbourne, Australia, 2019 (Represented by Dr. Cornelia Landersdorfer).

7. Bilal H, Bergen PJ, Tait JR, W SC, Peleg AY, Roberts JA, Oliver A, Nation RL, Landersdorfer CB. Clinically relevant epithelial lining fluid concentrations of meropenem with ciprofloxacin provide synergistic killing and resistance suppression of hypermutable *Pseudomonas aeruginosa* in a dynamic biofilm model. Poster presentation at the Australian Pharmaceutical Science Association (APSA) Annual Conference, Melbourne, Australia,1-4th Dec, 2019.

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Last but not least, I would like to thank 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 a desire to keep learning.

List of figures

List of abbreviations

AMR	Antimicrobial Resistance
MDR	multidrug-resistant
WHO	World Health Organization
MRSA	Methicillin-resistant *Staphylococcus aureus*
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species
ICU	Intensive care unit
IDSA	Infectious Diseases Society of America
CDC	Centers for Disease Control and Prevention
FDA	United States Food and Drug Administration
VAP	Ventilator-associated pneumonia
CRPA	carbapenem-resistant *P. aeruginosa*
APACHE II	Acute Physiology and Chronic Health Evaluation II
CFTR	CF transmembrane conductance regulator
FEV1	forced expiratory volume at 1 second
MMR	mismatch repair
ECM	extracellular matrix
EPS	exopolysaccharides
AHL	*N*-acyl-L-homoserine lactones
PQS	Pseudomonas quinolone signaling
PBPs	penicillin binding proteins
ROS	reactive oxygen species
ATCC	American Type Culture Collection
AUC	area under the concentration-time curve
cfu or CFU	colony forming units
CLSI	Clinical and Laboratory Standards Institute
EUCAST	European Committee on Antimicrobial Susceptibility Testing
C_{max}	maximum plasma concentration
C_{min}	trough plasma concentration
$\%T_{>MIC}$	cumulative percentage of a 24-h period that the drug concentration exceeds the MIC at steady state
$t_{1/2}$	half-life
PAE	post-antibiotic effect
SCTK	static concentration time-kill
ESBL	extended-spectrum beta-lactamase

UTI	urinary tract infection
G6P	glucose-6-phosphate
UhpT	hexose-phosphate (glucose-6-phosphate [G6P]) uptake system
GlpT	ı-α-glycerophosphate (glycerol-3-phosphate [G3P]) system
MurA	uridine diphosphate (UDP)-*N*-acetylglucosamine enolpyruvyl transferase
HFIM	Hollow fibre infection model
IVM	*in vitro* model
i.v.	intravenous/intravenously
V_d	volume of distribution
CL	clearance
L/h	litre/hour
INH	inhalation
mg/L	milligram/litre
MIC	minimum inhibitory concentration
ELF	epithelial lining fluid
PK/PD	pharmacokinetic/pharmacodynamic
CBR	CDC biofilm reactor

Chapter 1

Introduction

1. Statement of the problem

1.1 Antimicrobial resistance (AMR): A global health crisis

Antibiotics were one of the most impactful discoveries of the 20th century and transformed modern medicine, and were once regarded as 'Magic Bullets' (1, 2). The antibiotic era revolutionised infectious diseases treatment, saved millions of lives, shifted the death toll from communicable diseases to non-communicable diseases (*e.g.* cardiovascular, cancer and diabetes) and increased average life expectancy across the globe (3, 4). Consequently, it was presumed that the era of deadly infections had eventually come to an end (5). However, over the last several decades a significant increase in resistance of pathogens to almost all clinically available antibiotics, so-called multidrug-resistant (MDR) 'superbugs', has dramatically changed the global health scenario (6, 7). MDR bacteria are those that are resistant to more than one antibiotic in three or more antibiotic categories (8).

The World Health Organization (WHO) has announced AMR as one of the three greatest threats to human health (9, 10). The problem is considered such a threat that a post-antibiotic era, in which common infections and minor injuries could prove fatal, is quite possible in the 21st century (11, 12). Recently, it was estimated that ~0.7 million people die annually due to antimicrobial-resistant infections. More alarmingly, the number of people who will die of infections due to drug-resistant pathogens, could rise to 10 million per year by 2050 (Figure 1), if necessary measures are not undertaken to reduce the burden of these 'superbugs' (13).

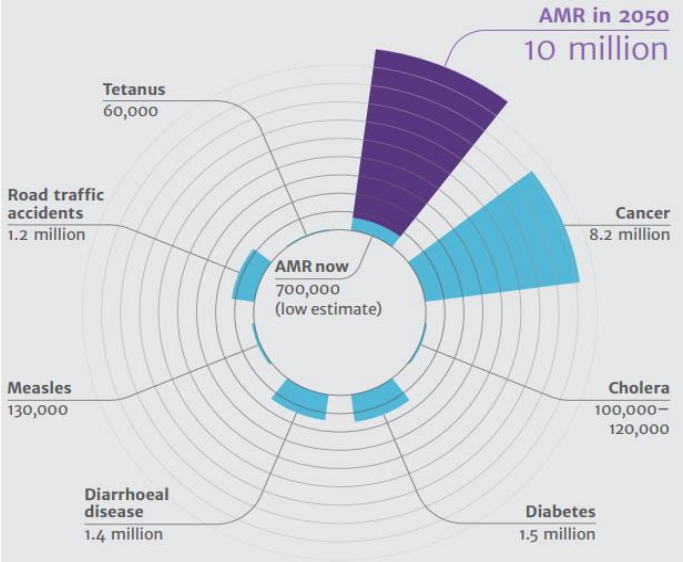

Figure 1: Deaths attributable to antimicrobial resistance (AMR) every year compared to other major causes of death. The figure shows diagrammatically the estimated number of deaths in 2014 (inner concentric circle) and in 2050 (outer concentric circle). From, 'Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations', Dec 2014 (13).

Several factors have played an integral role in the evolution of MDR bacteria and the weakening of the global therapeutic antibacterial armamentarium. Firstly, antibiotics have been used inappropriately and over-prescribed for many years, resulting in their reduced effectiveness against these 'superbugs' (6, 14). Under extreme selective pressures, bacteria employ multiple resistance strategies in order to survive exposure to antibiotics (15). Such strategies often include mutational alteration of target site proteins, acquisition of genes for less-susceptible target proteins, bypassing the target, preventing drug access to the targets, and enzymatic inactivation of the drug (16, 17). Secondly, despite the fact that the spread of antibiotic-resistant bacteria poses a substantial threat to morbidity and mortality worldwide, pharmaceutical research and development has failed to meet the clinical need for new antibiotics which are effective against resistant bacteria (18, 19). This is due, in part, to complex and lengthy regulatory processes involved in drug development, expensive and challenging post-marketing surveillance and low economic incentives for developing antibiotics that are typically given for short durations (20, 21). Furthermore, around the beginning of this century

the focus of new antibiotic development was largely directed towards Gram-positive bacteria, *e.g.* methicillin-resistant *Staphylococcus aureus* (MRSA) (22). Unfortunately, research into the treatment of infections caused by MDR Gram-negative bacteria, including *P. aeruginosa*, received less attention (23). Overall, the suboptimal use of currently available antibiotics, coupled with gaps in research and development of new antibiotics have significantly contributed to the rise in AMR, potentially paving the way for the dawn of a post-antibiotic era.

1.2 'Superbugs' need new treatments

The main contributors to antibiotic resistance are a group of pathogens known as the ESKAPE pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species (23). These bacteria are responsible for the majority of nosocomial infections and are known to 'escape' the bactericidal effects of many antibiotics *via* multiple drug resistance mechanisms (24). Four of the ESKAPE pathogens are Gram-negative bacteria (*K. pneumoniae, Acinetobacter* species, *P. aeruginosa*, and *Enterobacter* species); and three of these (*Acinetobacter* species, *P. aeruginosa*, and *Enterobacter* species) are on the WHO priority list, underlining the urgent need for new antibiotics and better use of existing antibiotics (25, 26).

Indeed, WHO has categorised carbapenem-resistant *P. aeruginosa* as a Priority 1: CRITICAL bacterial pathogen that requires the most urgent research and development for effective new treatments (27). *P. aeruginosa,* which is the main focus of this thesis, is responsible for the majority of nosocomial infections in intensive care units (ICUs) (28, 29). The Infectious Diseases Society of America (IDSA) has identified *P. aeruginosa* as one of the top six pathogens threatening healthcare systems, and the US Centers for Disease Control and Prevention (CDC) put it under the threat level 'serious' (25, 30). In the US, 13-19% of healthcare-associated infections (HAIs) each year, have been attributed to MDR *P. aeruginosa* (8, 31). Furthermore, in Europe many *P. aeruginosa* clinical isolates are resistant to multiple

anti-pseudomonal antibiotics (Figure 2), and the prognosis of patients in ICUs with such infections is very poor with a mortality rate as high as high as 50-60% (32, 33). Moreover, in Australian clinics, 31-35% of respiratory infections in patients with cystic fibrosis (CF) have been associated with MDR *P. aeruginosa;* poor prognosis and respiratory failure are associated with early death (34, 35). No doubt, the situation is quite overwhelming and a promising solution is urgently required. The US Food and Drug Administration (FDA) has approved a small number of new antibiotic products during the last decade, *e.g.* ceftolozane-tazobactam, ceftazidime-avibactam, meropenem-vabrobactam, imipenem-cilastin-relebactam and plazomicin, for clinical use against infections caused by MDR Gram-negative bacteria including *P. aeruginosa.* However, these products are not available in all countries and caution is warranted to retain their long-lasting effectiveness (36-40).

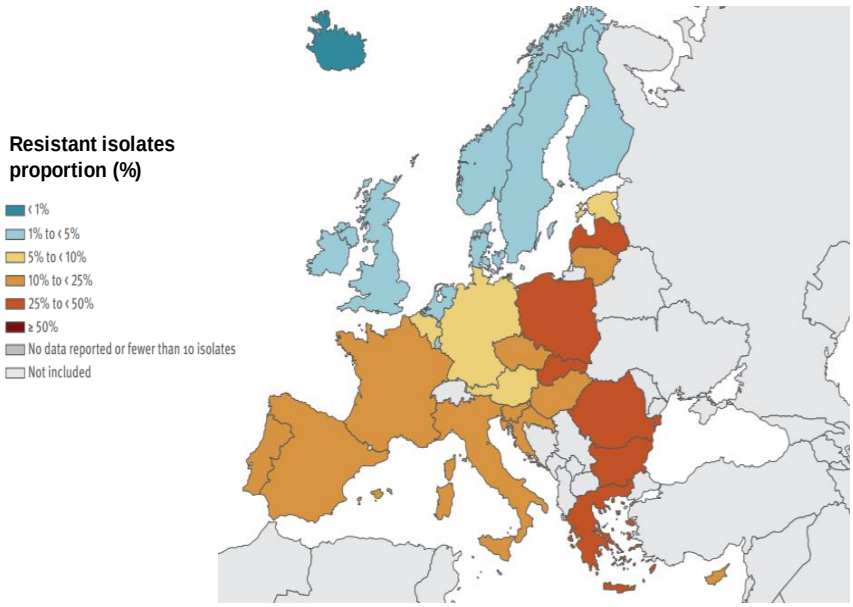

Figure 2: *P. aeruginosa* percentage (%) of resistant isolates with combined resistance (resistance to three or more antipseudomonal antibiotics among ceftazidime, piperacillin/tazobactam, fluoroquinolones, aminoglycosides and carbapenems). The data are for 2018, from the European Centre for Disease Prevention and Control (41).

1.3 *Pseudomonas aeruginosa*

P. aeruginosa is a ubiquitous, opportunistic pathogen, sometimes found on normal human skin, that is able to cause recalcitrant infections, especially in immunocompromised and hospitalised patients (42, 43). As mentioned above, it is common in settings such as hospitals and aged-care facilities and is responsible for a variety of infections, including those of the upper and lower airways, bloodstream, urinary tract, bone, joint, skin, soft tissue and eye (44). Such infections are potentially devastating particularly in patients with underlying conditions such as CF, the immunocompromised and the critically ill (45, 46). These infections are responsible for increased lengths of hospital stay, severe illness, need for surgical intervention, increased mortality and economic burden (47, 48).

The pathogenic profile of *P. aeruginosa* is related to its complex genome and a large and variable arsenal of virulence factors (49, 50). The outer membrane of *P. aeruginosa* has a low permeability (1/100th of the permeability of the *Escherichia coli* outer membrane) and acts as a barrier to antibiotics, and the pathogen has efficient efflux systems which expel antibiotic molecules from the bacterial cell (51). In addition, its versatile mode of growth, including capacity to form biofilms, provides *P. aeruginosa* an advantage in establishing infections, including ventilator-associated pneumonia (VAP) and CF lung infections, within susceptible hosts (49). During the last two decades, *P. aeruginosa* strains that display a hypermutator phenotype (*i.e.* an increased mutation rate) have been isolated from chronic respiratory infections of patients with CF (52-54). The presence of hypermutable *P. aeruginosa* in association with biofilm formation renders *P. aeruginosa* infections difficult to treat and often results in treatment failure (52, 55-57).

1.3.1 *P. aeruginosa* infections in critically ill patients: risk factors, significance, and associated mortality

The risk factors associated with the acquisition and establishment of *P*. *aeruginosa* infections in ICU are variable from one study to another and include: length of hospitalisation, severity of underlying disease, exposure to invasive procedures, contamination of tap water, close contact with contaminated patients and antibiotic selective pressure (58-60). The heterogeneity of the factors reported is due partly to the complexity of the measurement of exposures to these factors, the definition of the outcome and of the methods used for the analysis of the association with *P*. *aeruginosa* colonisation or infection (59).

P. aeruginosa causes catastrophic infections in critically ill patients (61). In ICUs, major infections are respiratory (very commonly VAP), central venous catheter-related bacteraemia, non-catheter related bacteraemia, secondary peritonitis, tracheostomy-related, surgical wound, burns and those within the urinary tract (62). These infections are associated with high mortality especially when infection is caused by MDR bacteria (62). A retrospective study evaluating 54 ICU patients with *P. aeruginosa* infections found that MDR *P. aeruginosa* was observed in 37% of cases (20 of 54 patients) and occurred in patients hospitalised for an average of 87.1 days (63). In a case control surveillance study, the prevalence of MDR *P. aeruginosa* was 54% among patients with *P. aeruginosa* infection with tracheal intubation; MDR strains were associated with a longer hospitalisation (39 *versus* 24 days) and a higher mortality (49% *versus* 20%) compared to non-MDR strains (64).

A large multicentre cohort study of 740 patients with *P. aeruginosa* nosocomial pneumonia demonstrated high (31%) prevalence of MDR isolates (65). The overall hospital mortality in that study was 35.7% and the mortality rate in MDR-infected patients was significantly higher (44.7% *versus* 31.7%) compared to non-MDR infected patients (65). While evaluating the risk factors for MDR and attributable mortality in ICU patients the same study demonstrated

decreasing age, diabetes mellitus and ICU admission as the independent predictive factors associated with MDR *P. aeruginosa* pneumonia (65). In another retrospective study, 63 episodes of carbapenem-resistant *P. aeruginosa* (CRPA) infections showed that the Acute Physiology and Chronic Health Evaluation II (APACHE II) score at the time of CRPA bacteraemia and the capacity of CRPA to form biofilm were independent predictive factors for mortality in patients with CRPA bacteraemia (66). In addition, the biofilm-forming ability and elastase activity of strains were correlated with APACHE II scores to measure the severity of disease and estimate predicted mortality in the patients. A systematic review of neutropenic patients with *P. aeruginosa* infections revealed an increased mortality (60%; 18 out of 30 studies) of patients with carbapenem-resistant *P. aeruginosa* strains compared to carbapenem-susceptible strains (67).

1.3.2 *P. aeruginosa* respiratory infections in patients with cystic fibrosis: risk factors, significance, and associated mortality

CF is a complex, life-limiting hereditary disease predominantly affecting the Caucasian population (68). One in 29 people of Caucasian ancestry is an unaffected carrier of the CF gene mutation (69). CF occurs at a rate of 1 in 2,500 births and about 70,000 to 100,000 people are affected worldwide (70, 71). This condition affects multiple systems of the human body due to a mutation in the CF transmembrane conductance regulator (CFTR) gene (68, 72, 73). The impaired functioning of the CFTR gene results in an inadequate production of CFTR protein, which in turn decreases the function of the mucus glands along with a poor self-regulated mucociliary clearance (73, 74). Thus, the airways in the lungs of people with CF become blocked by thick and sticky mucus (75). This dynamic environment in the respiratory tract of patients with CF serves as a favourable place for bacteria to grow, and therefore facilitates the acquisition of *P. aeruginosa* from the environment (76). Once *P. aeruginosa* is inside the airways, a toxic pro-inflammatory local microenvironment leads to a chronic inflammatory response as a result of virulence factors of *P. aeruginosa* (73). A healthy immune system has

phagocytic cells that ingest invading pathogens and limit infection. However, due to the presence of thick mucus and insufficiency of mucociliary clearance in patients with CF, *P. aeruginosa* resides in the airways for years by evading the host's immune responses and undergoes certain genotypic and phenotypic changes (77). Consequently, the initial infection often leads to the establishment of a chronic *P. aeruginosa* infection, which can ultimately lead to lung damage, pulmonary insufficiency, respiratory failure and eventually death (78).

Other than *P. aeruginosa* a range of other pathogens, including *S. aureus, Haemophilus influenzae* and *Burkholderia cepacia* are often found infecting CF lungs, however, *P. aeruginosa* is regarded as a major cause of morbidity and mortality in CF (79-81). *P. aeruginosa* infects approximately 60% of patients with CF overall, with an 80% prevalence in the group of patients above 18 years of age (82, 83). The median life expectancy of a patient with CF was 48 years in males and 43 years in females (84). However, patients with *P. aeruginosa* infection have a decreased life expectancy of 30 years, compared with 40 years in non-colonised patients, experiencing a more rapid decline in pulmonary function with more frequent hospitalisations (85-87). A study of 3,323 children aged 1-5 years revealed a 2.6-fold higher 8-year risk of death if *P. aeruginosa* was present in the lower airway (88). In addition, the clinical outcome data demonstrated both a lower forced expiratory volume at 1 second (FEV1) and a lower weight percentile. This increased risk continued if the child remained *P. aeruginosa* positive. Other studies also demonstrated an acceleration of lung disease in children with CF associated with *P. aeruginosa* infection (86, 87, 89). In a prospective study in a cohort of 56 children with CF identified by newborn screening, *P. aeruginosa* infection was common by age 7 years (43%) and was associated with increased morbidity and mortality (89). Another study reported that by the age of 3 years 97.5% of children with CF had a *P. aeruginosa* infection (90). When they reached adolescence, 80% of them had *P. aeruginosa* colonisation (79, 82). Other studies reported inappropriate treatment with non-antipseudomonal antibiotics and possible exposure to older *P. aeruginosa*-positive patients in

a CF clinic are potential risks for infants and young children acquiring *P. aeruginosa* in their lungs (91-93).

Other than the pathophysiological condition of the patient with CF, *P. aeruginosa* can also cause worsening of the disease through genotypic and phenotypic changes within the bacterial cell (89). As a result of various molecular changes that include hypermutation, decreased metabolism, reduced growth rate, decreased motility, alginate overproduction, biofilm formation, and efficient quorum sensing, treatment failure is often a negative outcome (57, 76, 94-96). These bacterial changes are often the result of life-long exposure to antibiotics and suboptimal dosing resulting in the emergence of resistance (89). Thus, once *P. aeruginosa* infection is established in the lungs of people with CF, it is extremely difficult to eradicate. In particular, the presence of the hypermutator phenotype and biofilm formation makes such infections extremely difficult to treat (52, 97), and such factors will be discussed in Sections 1.4 and 1.5 of this Chapter.

1.4 Hypermutable *P. aeruginosa*: its impact on disease progression

Some *P. aeruginosa* strains have the ability to increase their mutation rate rapidly through a process called hypermutation, thereby developing a hypermutator phenotype (54, 57, 98). Hypermutation occurs mainly through mutations within DNA repair pathways, especially in the mismatch repair (MMR) system (56). In non-hypermutators, the MMR system acts as a proof reader by recognising and repairing erroneous insertion, deletion, and mis-incorporation of bases that can arise during DNA replication and recombination, as well as by repairing some forms of DNA damage (99). However, in hypermutators this normal function is disturbed resulting in abnormal transcripts (98). Hypermutators have ~1000-fold increased mutation rate (Figure 3) which increases the probability of mutations occurring that confer antibiotic resistance, compared to non-hypermutators (57).

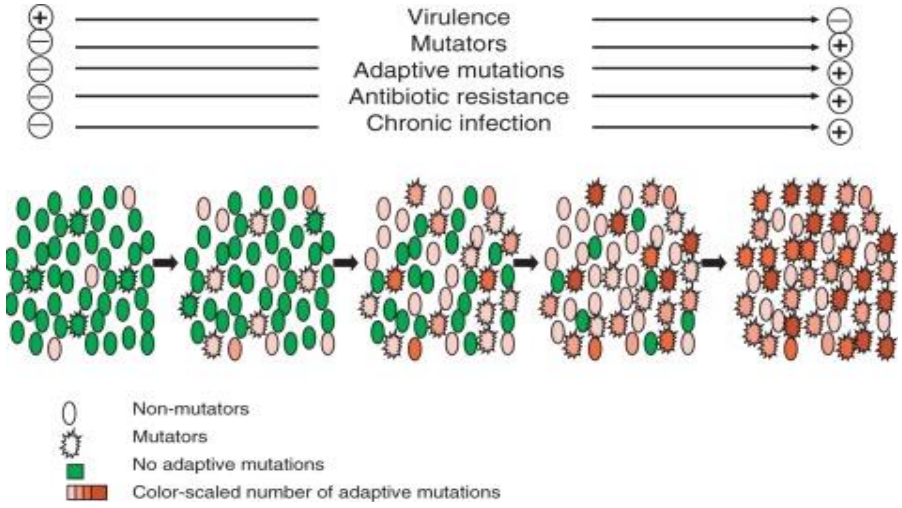

Figure 3: Schematic representation of the role of hypermutators as catalysers of adaptive evolution of *P. aeruginosa* in chronic lung infections of patients with CF. Figure reproduced with permission (100).

In *P. aeruginosa,* hypermutation often occurs as a result of a defective/non-functional *mutS* gene (98). Mutations in other genes, *i.e. mutL, mutU, mutY, mutM, mutT* and *mutD*, could also lead to a hypermutator phenotype (101). Hypermutable *P. aeruginosa* were first identified in 2000 from sputum samples of CF patients with chronic respiratory infections (54). Since their identification, such strains have been frequently isolated from CF patients and are associated with reduced lung function, worsened disease progression and respiratory failure (52, 53, 98). Hypermutators account for up to ~65% of *P. aeruginosa* strains in CF and are recognised as a major problem for antimicrobial therapy (54, 98). Despite increased mutation rates and short generation times, such strains generally do not show reduced fitness in the nutrient-rich environment of the CF lung but have a lower biological cost for some antibiotics (52, 102). Carriage of *P. aeruginosa* hypermutator strains is highly correlated with MDR and establishment of chronic infections through biofilm formation (103-105).

1.5 Biofilm formation: its role in establishment of chronic respiratory infection

Bacterial biofilms are a serious global health concern due to the ability of the bacteria within to tolerate antibiotics, host immune systems and other external stresses, thereby making infections more complex, difficult to treat and worsening the disease condition (106, 107). Biofilms are structured immobile microbial communities which colonise and grow on surfaces of lungs, medical implants and catheters, and enable shedding of bacterial cells to sustain the pool of planktonic bacterial cells to prolong infections (95). Biofilms are comprised of the bacterial population encased in extracellular matrix (ECM) which consists of bacterial secreted polymers such as exopolysaccharides (EPS), extracellular DNA (e-DNA), proteins and amyloidogenic proteins (108).

Biofilm formation is a self-regulated multi-step event (Figure 4) involving: i) initial attachment of mobile bacterial cells to the surface; ii) irreversible adhesion of bacteria, which form a monolayer along the surface; iii) secretion of a complex mixture of carbohydrates, proteins and lipids to encapsulate the bacteria; (iv) colony formation and biofilm maturation and, v) biofilm dispersion upon signal from the environment (waste build up or demand for nutrients) (49, 52, 108, 109). Biofilm bacteria present an altered growth rate as compared to the planktonic bacteria due to decreased metabolic activity associated with reduction of nutrients and oxygen levels deep within the matrix. (49, 110). Biofilm bacteria are a complex community of cells capable of communicating *via* molecular signalling comprising the so-called quorum sensing system (105). While bacterial cells in the deeper layers are metabolically less active, those in the superficial layers of the biofilm may be released and may recover their planktonic properties; they may finally colonise and attach to new surfaces, thus extending the biofilm structure (111). Approximately 80% of chronic and recurrent microbial infections in the human body involve bacterial biofilms (112).

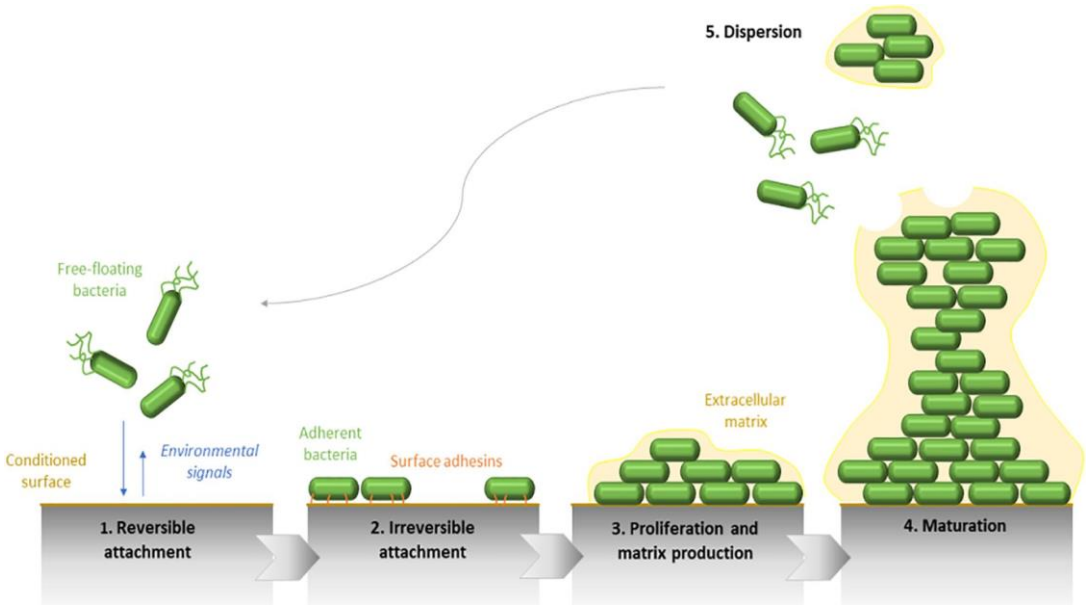

Figure 4: Schematic representation of the five main steps defining the *P. aeruginosa* biofilm development: i) initial attachment of mobile bacterial cells to the surface; ii) irreversible adhesion of bacteria, which form a monolayer along the surface; iii)secretion of a complex mixture of carbohydrates, proteins and lipids to encapsulate the bacteria; iv) biofilm maturation characterised by the matrix production and the formation of three-dimensional structures; and, v) biofilm dispersion upon signal from the environment (waste build up or demand for nutrients). Figure reproduced with permission(109).

P. aeruginosa biofilms are common and life threatening in CF patients and individuals with a compromised immune system (52). While the matrix of *P. aeruginosa* biofilms generally consists of polysaccharides, proteins, extracellular DNA and lipids, its composition is strain dependent, and also depends on the growth conditions and the age of the biofilm (113). In *P. aeruginosa* biofilms, quorum sensing which involves *N*-acyl-L-homoserine lactones (AHL) and *Pseudomonas* quinolone signaling (PQS) molecules are responsible for bacterial chemical communication and cell-to-cell signaling under restricted conditions (114, 115); however, it is important to note that this is not the only system that may be involved for *P. aeruginosa* adaptation in the lungs of people with CF (106, 113). Perhaps more important are the genes and pathways involved in the conversion of *P. aeruginosa* from its normal phenotype to hypermutator and mucoid phenotype (95, 110). This phenotypic shift is highly correlated with the establishment of chronic and tolerant *P. aeruginosa* infection (82).

There are certain biofilm-associated antimicrobial resistance mechanisms that play a role in developing tolerance to specific antibiotic classes, *e.g.* the primary tolerance mechanism of biofilms to β-lactams is related to the slow growth of bacteria in biofilms providing less target penicillin binding proteins (PBPs) (112). Overexpression of the β-lactamase enzyme AmpC is also a common mechanism of developing resistance to β-lactams in biofilms (116). Resistance of biofilm bacteria to aminoglycosides occurs *via* chelation to various components of the biofilm matrix, especially extracellular DNA (eDNA), as well as expression of specific genes that confer biofilm-associated aminoglycoside tolerance (117, 118). Although fluoroquinolones are generally more active than β-lactams against biofilm bacteria, the low oxygen concentration in biofilms affects the bactericidal activity of fluoroquinolones, probably due to formation of reactive oxygen species (ROS) in insufficient levels to cause bactericidal effects (118, 119). Hypermutable *P. aeruginosa* in association with biofilm formation renders difficult the treatment of respiratory infections, especially in people with CF, and often results in treatment failure and multidrug-resistance (103, 120).

1.6 Antibacterial treatments for *P. aeruginosa* infections

P. aeruginosa is intrinsically resistant to many antibiotics and, even with appropriately chosen antibiotics, treatment failure due to selection of resistant mutants is a substantial problem (121). The array of traditional antibiotics serving as the backbone of treatment of *P. aeruginosa* infections includes β-lactams, aminoglycosides and fluoroquinolones; fosfomycin has recently demonstrated significant activity against MDR *P. aeruginosa* isolates and there is substantial interest in its use for infections caused by this 'superbug' (122-124). These antibiotics inhibit or kill the bacteria by blocking various steps involved in bacterial growth, and such mechanisms for each antibiotic class will be discussed in Sections 1.6.1-4 of this Chapter. However, prior to those sections it is appropriate to briefly review general mechanisms of bacterial resistance to antibiotics and general principles of antibacterial pharmacodynamics (PD), pharmacokinetics (PK) and the interrelationship between PK and PD (*i.e.* PK/PD).

General mechanisms of resistance: *P. aeruginosa* has an extraordinary capacity to develop resistance to various antibiotics (116). It can also acquire multiple antimicrobial resistance genes encoded in plasmids and transposons through horizontal transfer from other Gram-negative bacteria (125-128). Some of the important resistance mechanisms involve reduced permeability of the outer membrane resulting in reduced intracellular antibiotic concentration, overexpression of multiple efflux pump systems, enzymatic cleavage/inactivation of antibiotics and antibiotic target site modifications, mechanisms whose impacts are enhanced through hypermutability and biofilm formation (44, 113, 115, 129). Common mechanisms of resistance of *P. aeruginosa* for each antibiotic class are represented diagrammatically in Figure 5.

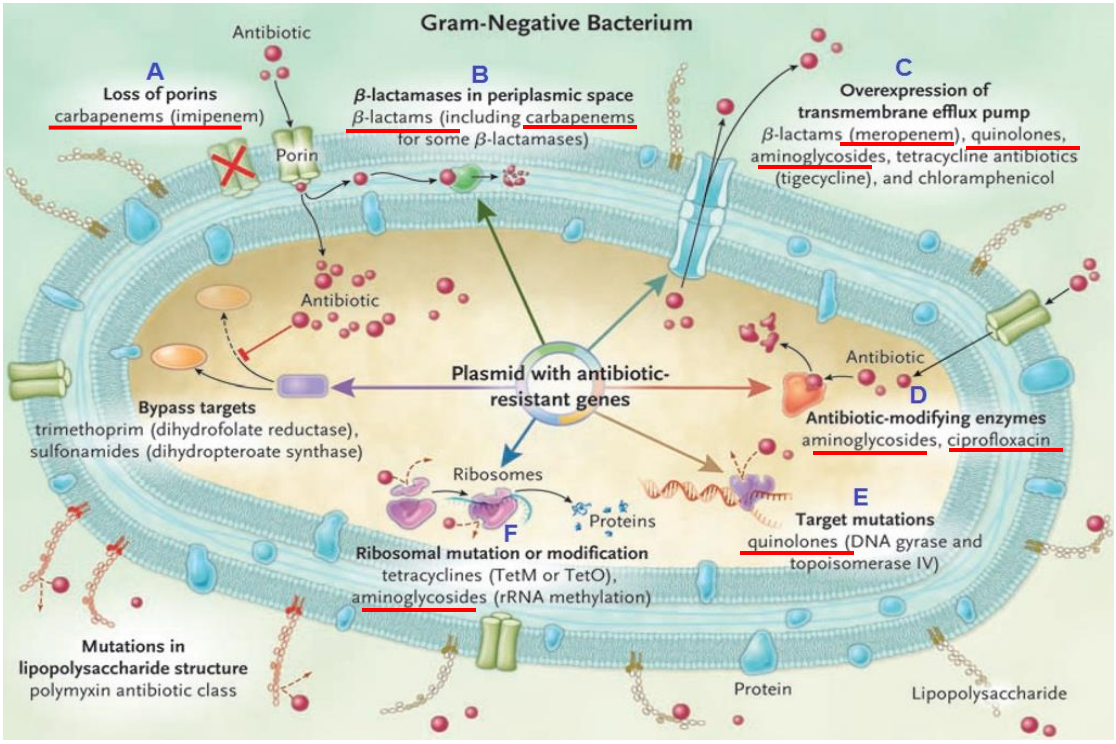

Figure 5: Mechanisms of antibiotic resistance in Gram-negative pathogens, including *P. aeruginosa* (130). Antibiotic classes relevant to this thesis (β-lactams, aminoglycosides and quinolones) are underlined in red. The resistance mechanisms for each of these classes and for the other antimicrobial agent relevant to this thesis (fosfomycin) are discussed in the Sections 1.5.1-4. Figure reproduced with permission, copyright Massachusetts Medical Society.

In addition to the mechanisms of action and resistance, it is important to have a solid understanding of the general principles of antimicrobial PD, PK and PK/PD when suggesting dosing schemes for the optimal use of antibiotics against infections, especially those that are difficult to treat.

General principles of PD: Antibacterial PD refers to the relationship between the concentration of an antimicrobial and its effect (131). The most common way to quantify the PD activity of an antibiotic against a particular pathogen has traditionally been the minimum inhibitory concentration (MIC), which is the lowest concentration of an antibiotic that inhibits the visible growth of a microorganism after overnight (18-24 h) incubation (132). The MIC is regarded as a measure of potency of an antibiotic, and varies depending on the antibiotic, the organism and its underlying resistance mechanisms against the antibiotic (132). Despite the MIC having been utilised for decades as a measure of susceptibility of an organism for a particular antibiotic, it is also very important to recognise its limitations which include; i) MIC is determined using a low inoculum that may not reflect many clinical infections; ii) MIC is simply a visual examination (*i.e.* 'turbid' *versus* 'not turbid') at a single point in time (*e.g.* 18-24 h); there are no counts of viable bacteria, and a lack of turbidity does not mean a lack of viable bacterial cells; iii) MIC measurement does not reveal information about the time-course of bacterial killing nor the time- or concentration-dependence of antibacterial activity of antibiotics; iv) MIC does not provide information about the possible presence of resistant sub-populations in the isolate; and, v) MIC is determined at a fixed time point after exposure to static antibiotic concentrations, hence it provides no information about post-antibiotic effect (PAE), which refers to the persistent suppression of bacterial growth as concentrations decrease from supra- to sub-MIC values as may occur with dosage regimens in a patient (133-135).

Greater insight into the PD behaviour of an antibiotic can be obtained from studies in which a bacterial inoculum is exposed to a range of static antibiotic concentrations, quite often expressed as a multiple of the MIC, and the number of viable bacterial cells per millilitre is

measured at various times. This is known as a static concentration time-kill (SCTK) study. On the basis of their pattern of killing, antibiotics are typically characterised in one of three ways: concentration-dependent killing with prolonged persistent effects; time-dependent killing with minimal-to-modest persistent effects; and, time-dependent killing with prolonged persistent effects (136). Time-dependent activity is characterised by saturation of the rate of killing with increasing concentrations, with higher concentrations not producing faster killing than lower concentrations (131, 137). Concentration-dependent activity is characterised by a greater rate and extent of killing with higher concentrations over a wide concentration range (131, 137). The SCTK profiles in Figure 6 show the effect of increasing concentrations on the *in vitro* antibacterial activity of three different antibiotics against a strain of *P. aeruginosa*. Increasing concentrations of tobramycin (an aminoglycoside) and ciprofloxacin (a fluoroquinolone) produced more rapid and extensive bacterial killing, whilst for ticarcillin (a β-lactam) higher concentrations resulted in earlier initiation of bacterial killing but did not increase the rate of killing after 2 h of treatment (135). The PD characteristics described here suggest that the time-course of antibacterial activity can vary markedly for different antibiotics and needs to be considered when designing dosage regimens.

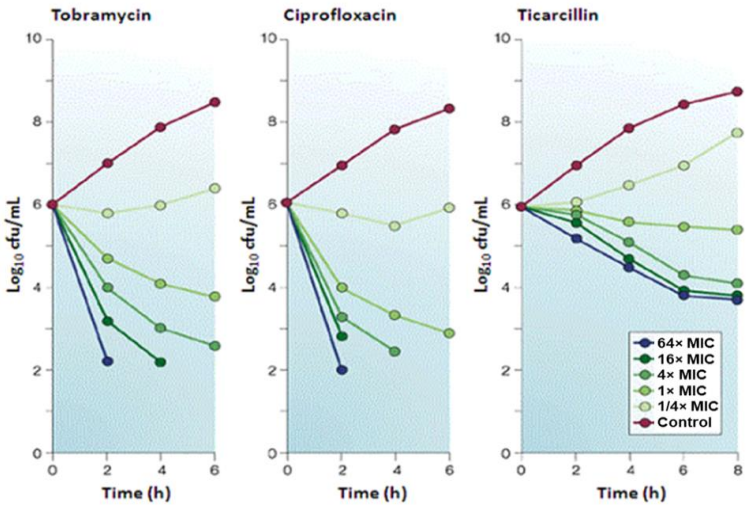

Figure 6: Pattern of bacterial killing against *P. aeruginosa* exposed to different antibiotics; tobramycin (left), ciprofloxacin (centre), and ticarcillin (right) at concentrations from ¼ to 64× MIC (135). Plots show colony forming units (CFU) per mL as a function of time. Figure reproduced with permission.

While SCTK studies can be very useful and are widely used because of their relative simplicity and financial advantage, they do not mimic the dynamic situation that occurs in a patient receiving a dosing regimen that results in fluctuating concentrations of an antibiotic. In PK/PD models bacteria can be exposed to dynamic (*i.e.* fluctuating) concentrations of antibiotic over time to mimic *in vivo* PK (138). Thus, data generated from dynamic PK/PD models more closely reflect the clinical situation than do data from a SCTK study. One-compartment *in vitro* PK/PD models are widely used. The study described in Chapter 2 of this thesis utilised such a model to characterise the PK/PD of fosfomycin against *P. aeruginosa*, while in Chapters 3 to 5 a modified form of the one-compartment model was used to examine the activity of combinations of various antipseudomonal antibiotics against both planktonic and biofilm bacteria. Detailed descriptions of the models are provided in those chapters.

PK properties: Antibacterial PK relates to the disposition (absorption, distribution, metabolism, and excretion) of an antibiotic; these factors determine the concentration-time profile of antibiotic in plasma or serum, tissues and body fluids following a dosage regimen (139). PK parameters (clearance (CL), volume of distribution (Vd) and half-life) of an antibiotic can vary significantly between various groups such as healthy volunteers, patients with CF and the critically ill (140, 141). As such, PK data from specific patient groups is important for designing preclinical *in vitro* and *in vivo* studies and also clinical investigations.

Interrelationship of PK and PD: The time-course of antibacterial activity reflects the inter-relationship between PK and PD. The PD characteristics from SCTK studies described above suggest that the time-course of antibacterial activity can vary markedly for different antibacterial agents (142). The use of PK/PD models enables characterisation of the exposure – response (*i.e.* PK – PD) relationship and identification of the so-called PK/PD index that most closely correlates with the antibacterial activity. There are three commonly used PK/PD indices (Figure 7), namely: the time that the unbound drug concentration (typically measured in plasma/serum) exceeds the MIC (or a multiple of MIC) over 24 h ($fT_{>MIC}$); the area under the

unbound (free) drug concentration-time curve over 24 h divided by the MIC (*f*AUC/MIC); and, the maximal unbound drug concentration divided by the MIC (fC_{max}/MIC) (143). These indices (Figure 7) have been found to be very informative for selecting effective dosage regimens and are capable of providing an indication of the likelihood of clinical outcomes (142, 144-146).

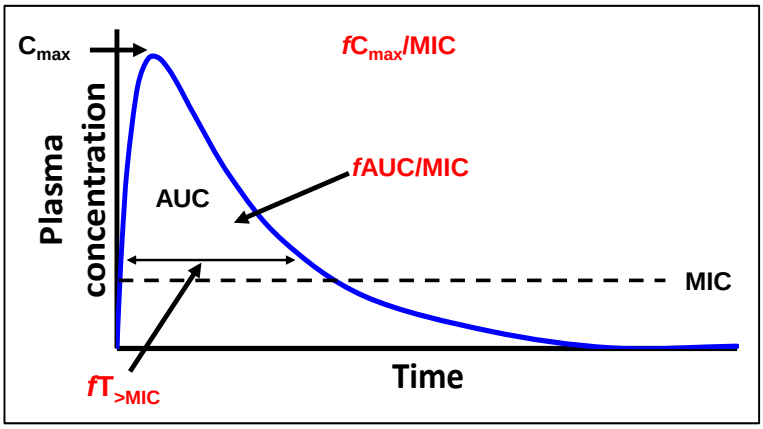

Figure 7: Relationship between the concentration-time profile, MIC and PK/PD indices (fC_{max}/MIC, *f*AUC/MIC and $fT_{>MIC}$) (147).

For each of the antibiotics included in studies in this thesis, the respective mechanisms of action and resistance, PD characteristics, PK properties and PK/PD indices are discussed in Sections 1.6.1-4.

1.6.1 β-Lactam antibiotics

β-Lactam antibiotics are frequently used to treat nosocomial infections in critically ill patients and respiratory infections in people with CF (148-151). Consequently, a large number of agents have been developed in this class. Members of this group share a common structure (β-lactam ring) in their molecule and exert their effect by inhibiting the synthesis of cell wall of *P. aeruginosa* (149, 152, 153). There are four classes of β-lactams including penicillin derivatives (penems), cephalosporins, monobactams and carbapenems (152). The penicillins include piperacillin (154) and the cephalosporins are divided into 1st, 2nd, 3rd, 4th and 5th generations. Currently, third generation cephalosporins are the most frequently used subclass

and include ceftazidime (155). The monobactam class has only one approved antibiotic, which is aztreonam (156) and the carbapenems include meropenem and imipenem (157).

Mechanisms of action: The β-lactam antibiotics inhibit the structural crosslinking of peptidoglycans in bacterial cell walls by binding to one or more specific penicillin-binding proteins (PBPs) which are a family of enzymes located in the periplasmic space and bound to the bacterial cytoplasmic membrane (158, 159). The peptidoglycan layer is essential for bacterial cell wall structural integrity. The binding of a β-lactam with PBPs ultimately leads to inhibition of peptidoglycan synthesis and finally cell death (160).

Mechanisms of resistance: The common mechanisms of resistance for β-lactam antibiotics are illustrated in Figure 5 and include modification of the PBP target sites, enzymatic inactivation by β-lactamases, active efflux *via* the MexAB-OprM, MexXY-OprM and MexCD-OprJ efflux pumps, (161, 162) and changes to the outer membrane protein type or loss of outer membrane proteins. The loss of the porin OprD results from the most prevalent mutation that confers resistance to meropenem and imipenem (163). *P. aeruginosa* can very often accumulate different resistance mechanisms, including production of extended spectrum cephalosporinases and metallo-β-lactamases leading to carbapenem resistance. All *P. aeruginosa* isolates have an inducible chromosomal AmpC β-lactamase that irreversibly modifies some β-lactams (164). Resistance to ceftazidime and aztreonam often occurs as a result of overexpression of AmpC (165). A number of studies have demonstrated that a β-lactam in monotherapy is not a viable option for *P. aeruginosa* and it leads to emergence of resistance (166-170).

PD: Several *in vitro* and *in vivo* studies demonstrated that β-lactam antibiotics exhibit time-dependent killing (135, 142, 171). β-Lactams show a saturation of the rate of bacterial killing (135). Thus, increases in concentration have minimal effect on the rate of killing and β-lactams often require longer antibiotic exposures to demonstrate antibacterial effects (172).

PK: Clinical pharmacokinetics of meropenem and ceftazidime (included in studies in this thesis) are discussed in this section. For meropenem, studies in healthy volunteers reported the volume of distribution at steady-state (V_{ss}), clearance (CL), and half-life to be 15-20 L, 11-19.7 L/h and ~1 h, respectively (173, 174). The plasma protein binding of meropenem is low, being only ~9% (173). The area under the plasma concentration-time curve (AUC) is linear over the dose range 0.25 to 1 g (173). Bui *et al.* reported no significant difference in pharmacokinetic data between clinically stable patients with CF and non-CF populations, after receiving 2 g of meropenem every 8 h (175). Meropenem is predominantly eliminated as unchanged drug in urine and as a result its total body clearance is directly related to renal function. In patients with decreased renal function the dosage must be reduced accordingly (166, 176). The distribution of meropenem is primarily extracellular. Its concentration in body fluids, such as epithelial lining fluid (ELF) in the case of lung infections, is considered to be more relevant than that in plasma. Meropenem displays a wide variability in ELF/plasma penetration ratio, ranging from 30-65% in diverse groups (174, 177-179). A study by Lodise *et al.* in patients with VAP exploring PK in plasma and ELF demonstrated relatively low peak concentrations in the ELF but terminal half-life was similar to that in plasma. An AUC_{ELF}/AUC_{plasma} penetration ratio derived from the mean parameter vector from the population model was 30% (177). In another study, Wenzler *et al.* demonstrated relatively high peak concentrations of meropenem in the ELF and an AUC_{ELF}/AUC_{plasma} penetration ratio of 65% (174). The effect of different levels of ELF penetrations of meropenem on bacterial killing has been explored in Chapters 3 and 4 of this thesis.

For ceftazidime, the mean total apparent volume of distribution is between 15 and 20 L in healthy volunteers with normal renal function and the elimination half-life is usually between 1 and 3 h (180). Systemic clearance is reduced with age, mainly as a result of decreased renal function, so dose adjustment is required in patients with renal impairment (181). Ceftazidime is a widely used antibiotic particularly for the treatment of *P. aeruginosa* infections in patients

with CF (182). In this group of patients both volume of distribution and renal clearance are increased. This has been attributed to fever-induced increases in renal blood flow. However, in more severely ill patients who do not have CF renal blood flow as a fraction of cardiac index, is not increased (183). Bulitta *et al.* reported a population PK model-informed total clearance of 7.82 L/h for CF patients and 6.68 L/h for healthy volunteers (184). For patients with respiratory infections, antibiotic concentrations in ELF are clinically relevant. Like other β-lactams, ceftazidime displays a wide variability in ELF/plasma penetration ratio; concentrations of ceftazidime in ELF were on average ~20%–53% of those in plasma and the ELF/plasma concentration ratio was not influenced by dose (185-188).

PK/PD: Studies have been conducted to identify the PK/PD index that best predicts the efficacy of various β-lactam antibiotics. Traditionally the most predictive PK/PD index driving the antibacterial activity is the duration of the dosing interval over which the unbound concentration remains above the MIC, or a multiple of it, of the infecting pathogen ($fT_{>n \times MIC}$) (189-192). *In vitro* and *in vivo* experiments have shown that the $fT_{>MIC}$ should be ≥50%, ≥60-70%, and ≥40% of the dosing interval for penicillins, cephalosporins, and carbapenems, respectively, to achieve at least 2 $\log_{10}$ bacterial killing at 24 h (142, 145). For serious bacterial infections, targets such as 100% $fT_{>4\text{-}5xMIC}$ have been suggested for resistance suppression and clinical success (192).

1.6.2 Aminoglycosides

Aminoglycosides are among the most commonly used broad-spectrum antibiotics in the antimicrobial armamentarium (193, 194). The aminoglycosides are bactericidal, and have predictable PK and a narrow therapeutic window (193). Despite the potential risk for renal toxicity, ototoxicity, and bacterial resistance, several members of this family of antibiotics have enjoyed clinical use for several decades and remain important in the treatment of infections caused by *P. aeruginosa* (171, 195).

Mechanisms of action: Aminoglycosides inhibit protein synthesis by binding, with high affinity, to the A-site on the 16S ribosomal RNA of the 30S ribosome (193, 196). As a result of this interaction, the antibiotic promotes mistranslation by inducing codon misreading on delivery of the aminoacyl transfer RNA (197). This results in error prone protein synthesis, allowing for incorrect amino acids to assemble into a polypeptide that is subsequently released to cause damage to the cell membrane and elsewhere (196, 197). Some aminoglycosides can also impact protein synthesis by blocking elongation or by directly inhibiting initiation (193).

Mechanisms of resistance: Aminoglycoside resistance in *P. aeruginosa* commonly occurs *via* enzymatic inactivation by aminoglycoside modifying enzymes (194). The enzymatic inactivation of aminoglycosides intracellularly occurs *via* interaction with acetyltransferases, phosphoryltransferases or adenyltransferases that acetylate, phosphorylate or adenylate the aminoglycosides, respectively (198, 199). Other mechanisms of resistance include target site modification *via* an enzyme 16S rRNA methyltransferases or chromosomal mutation, and reduced outer membrane permeability (116, 200). Aminoglycoside resistance in *P. aeruginosa* also occurs *via* the up-regulation of the MexXY-OprM efflux pump (200, 201). The up-regulation of this efflux pump frequently produces adaptive resistance from the over-expression of the MexY component during treatment (202). The use of aminoglycosides in monotherapy against *P. aeruginosa* infections often fails due to rapid emergence of resistance as a result of adaptive resistance (201, 203). However, aminoglycosides synergise with a variety of other antibiotics to enhance bacterial killing and improve the safety and efficacy of the class through optimised dosing regimens (166, 168, 204).

PD: Unlike β-lactams the antibacterial activity of aminoglycosides is found to be proportional to their concentration, with more rapid and extensive bacterial killing observed at higher concentrations (205). Aminoglycosides typically produce a prolonged post-antibiotic effect (PAE) (206, 207). PAE has been shown to be directly related to the length of time that the bacteria take to recover from the inhibition of protein synthesis and it has been proposed in the

past that a PAE may suppress susceptible populations from regrowing (208-210). The PAE duration is also related to the antibiotic concentration, duration of antibiotic exposure and bacterial density (209, 211, 212).

PK: The clinical pharmacokinetics of tobramycin, the aminoglycoside most commonly used in patients with CF and also the member of this class studied in this thesis, will be discussed here. The recommended standard dose of tobramycin for critically ill patients with normal renal function is 5 mg/kg given once daily with a maximum dose of 7 mg/kg/day as a 0.5 h infusion resulting in peak plasma concentrations of ~15-30 mg/L (213-215). However, for patients with CF the FDA has recommended a dose of 10 mg/kg/day (216), and Hennig *et al.* suggested a dose of 11 mg/kg/day may be optimal for CF patients (217). The population PK of tobramycin has been well described by two-compartment, linear models (217, 218). Tobramycin has a half-life of 3-4 h in critically ill patients (213, 219). Tobramycin has very low or no plasma protein binding, and therefore it freely distributes into the interstitial or extracellular fluid (220). The reported average clearance and volume of distribution of tobramycin were 3.83 L/h and 25.5 L, respectively, in ICU patients (213), whereas Hennig *et al.* reported an increased clearance of 9.5 L/h in patients with CF (218). Carcas *et al*. reported an AUC_{ELF}/AUC_{plasma} penetration ratio of tobramycin obtained from 16 patients with pneumonia of approximately 50 % (221).

PK/PD: Studies have shown that the PK/PD indices that are most often correlated with efficacy of aminoglycosides are *f*AUC/MIC and fC_{max}/MIC (142). An *f*AUC/MIC of >70 and an fC_{max}/MIC of 8-10 have been proposed as clinical targets (146). These targets can be achieved for clinical benefits by administering tobramycin once daily compared to three times daily dosing, also to reduce the renal toxicity (222). Therefore, clinical dosing regimens to optimise the probability of achieving the desired response should attempt to maximise the fC_{max}/ MIC, at any given *f*AUC/MIC value, by extending the dosing interval to prevent accumulation and toxicity (166). We have applied the same strategy in Chapters 3 and 5 to maximise the bacterial killing and suppress emergence of resistance.

1.6.3 Fluoroquinolones

Fluoroquinolones are an important class of broad-spectrum antibacterial agents. There are four generations of fluoroquinolones with nalidixic acid, which lacks a fluorine atom considered as first generation, being introduced for clinical use in 1962 (223). Nalidixic acid showed a narrow spectrum of activity and was initially restricted to treat Gram-negative urinary tract infections (224). Subsequently, the molecular structures of quinolones were modified to improve their antimicrobial properties, spectrum and PK profiles (225-227). Ciprofloxacin, one of the most commonly used fluoroquinolones, was introduced into clinical practice in 1987 (228); it is the agent from this class that was examined in this thesis.

Mechanisms of action: Fluoroquinolones inhibit DNA replication by blocking the activities of DNA gyrase (topoisomerase II) and topoisomerase IV, two enzymes essential for bacterial replication (229). DNA gyrase is an enzyme found only in bacteria and utilises the energy from ATP hydrolysis to introduce negative supercoils into DNA which is essential for chromosome condensation, relieving torsional strain during replication; whereas topoisomerase IV is required at the terminal stages of DNA replication for unlinking newly replicated daughter chromosomes (229, 230). The use of a low fluoroquinolone concentration results in the impairment of DNA replication whilst higher concentrations lead to cell death (142, 189).

Mechanisms of resistance: Fluoroquinolone resistance in *P. aeruginosa is* related to: (i) chromosomal mutations in genes encoding the target protein structures, lowering the binding affinity, or mutations causing reduced drug accumulation, either by a decreased uptake or by an increased efflux, and (ii) plasmid-located genes associated with quinolone resistance (231). Such mutations result in raised MIC (232). High-level ciprofloxacin resistance has been observed following exposure to ciprofloxacin concentrations below the MIC and overexpression of efflux pumps, *i.e.* MexAB-oprM, MexCD-OprJ (161, 233).

PD: Similar to aminoglycosides, fluoroquinolones are classed as concentration-dependent antibiotics, with higher concentrations associated with more rapid bacterial killing. The *f*AUC and the fC_{max} are often correlated with clinical and microbiological success in patients (135, 234). Rees *et al*. have demonstrated that high peak concentrations produced more pronounced microbiological response than regimens with lower peaks at the same *f*AUC (235).

PK: The clinical pharmacokinetics of ciprofloxacin have been extensively evaluated in stable CF patients (236-238). A study in 12 CF patient received a 400 mg intravenous dose of ciprofloxacin, reported the average volume of distribution at steady-state (V_{ss}), clearance (CL), and half-life to be 1.1 L/kg, 0.8 L/h/kg and 2.9 h, respectively (239). Ciprofloxacin disposition in patients with CF was well explained by a two-compartment population PK model (239). This study reported a lower clearance and smaller volume of distribution than the previous studies in patients with CF and which was regarded as being due to differences in age and disease maturity (236-238). A study by Forrest *et al*., reported similar PK results in acutely ill, non-CF patients as observed in patients with CF by Montgomery *et al.* (240). Schuler *et al*. studied intrapulmonary PK after a single oral dose (500 mg) of ciprofloxacin in 15 patients undergoing diagnostic bronchoscopy and bronchoalveolar lavage, and reported that the median concentrations in the ELF and plasma were 2.11 mg/L and 2.33 mg/L, respectively, at 2.5 h (241). In another study in 12 healthy volunteers, Gotfried *et al*. administered 500 mg ciprofloxacin orally every 12 h for 9 doses, and reported ELF concentrations of 1.9 ± 0.9 mg/L and 0.4 ± 0.1 mg/L at 4 h and 12 h, respectively (242).

PK/PD: Antibacterial activity of ciprofloxacin has been correlated with the magnitude of the *f*AUC/MIC and the fC_{max}/MIC ratios (142, 144, 191). In acutely ill patients with bacterial infections, an AUC/MIC of 125 (corresponding to an *f*AUC/MIC of 87.5) and a C_{max}/MIC of ≥8 (fC_{max}/MIC ≥5.6) have been proposed as targets to maximise the probability of clinical and microbiological cure (146, 234, 243).

1.6.4 Fosfomycin

Fosfomycin was discovered in 1969 (244). It is a phosphonic acid antibacterial agent (245). Fosfomycin has a broad spectrum of activity against Gram-positive and Gram-negative organisms including many MDR species (246-249). *In vitro* studies indicate fosfomycin remains active against many of the most problematic pathogens such as *P. aeruginosa*, including a minor but important subset of MDR *P. aeruginosa* (250, 251). Fosfomycin has been used primarily to treat urinary tract infections, where reports of resistance to fosfomycin are rare despite many years of use (252-254).

Mechanisms of action: Fosfomycin disrupts bacterial cell wall formation by interfering with peptidoglycan synthesis (255). Fosfomycin acts as a phosphoenolpyruvate (PEP) analogue and irreversibly inhibits uridine diphosphate-*N*-acetylglucosamine enol-pyruvyl transferase (MurA), which catalyses the condensation of uridine diphosphate-*N*-acetylglucosamine with PEP (256, 257). This is the initial step of cell wall synthesis involving phosphoenolpyruvate synthetase (245, 258). Thus, the synthesis of peptidoglycan is inhibited by blocking the formation of *N*-acetylmuramic acid resulting in cell lysis and cell death (245, 259).

Mechanisms of resistance: Mechanisms responsible for fosfomycin resistance include: reduced antibiotic uptake into bacterial cells, modifications of the site of action (MurA) and inactivation of the antibiotic by plasmid encoded enzyme FosA (255, 259-263). Fosfomycin enters bacterial cells by two different transport uptake systems; a constitutively functional L-α-glycerophosphate transporter (GlpT) and the hexose–phosphate uptake system (UhpT) (245). Both GlpT and UhpT are present in *E. coli* and numerous *Enterobacterales*, whereas only GlpT is present in *P. aeruginosa* (255, 264) and any mutation to the *glpT* gene leads to reduced intracellular concentrations of fosfomycin which result in emergence of resistance.

PD: Well-defined PD properties of an antibiotic play an important role in designing optimal dosing regimens. Some studies suggested that bacterial killing by fosfomycin is categorised

pharmacodynamically as time-dependent (265-267). Fransen *et. al.* also reported time-dependent killing activity of fosfomycin, but a key finding of this study was that the killing behaviour of fosfomycin not only depended on the bacterial species but also strain within a species (268). Although fosfomycin use for MDR *P. aeruginosa* infections has increased dramatically, currently there are no fosfomycin clinical breakpoints for *P. aeruginosa* to guide interpretation of susceptibility testing results. The existing Clinical and Laboratory Standards Institute (CLSI) (269) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) (270) MIC breakpoints are only for *Enterobacterales* and are not used for *P. aeruginosa* due to much higher wild type MICs of *P. aeruginosa* than *E. coli.*

PK: While exploring the clinical PK of intravenous fosfomycin, Merino-Bohórquez *et al.* reported high variability of fosfomycin concentrations among 64 samples from 16 patients after 4 g every 6 h and 8 g every 8 h, a finding partially explained by various degrees of renal impairment across the patients in the study (271) suggesting an important role for renal excretion in the total clearance. This was supported in a study involving serial samples from 28 healthy volunteers following intravenous administration of a single 1 or 8 g dose of fosfomycin, wherein the clearance was 8.7 ± 1.7 L/h and 7.8 ± 1.4 L/h, respectively, and with both doses the majority was contributed by renal clearance (272). In that study, the corresponding values of volume of distribution (Vd) were 29.7 ± 5.7 L and 31.5 ± 10.4 L, while those of half-life were 2.4 ± 0.4 h and 2.8 ± 0.6 h. A population PK study by Parker *et al*. reported significant PK variability of fosfomycin in critically ill patients which was correlated with observed variations in renal function (273).

PK/PD: Antibacterial activity of fosfomycin has been correlated with the magnitude of the *f*AUC/MIC against various Gram-negative bacteria. From studies conducted in a one-compartment infection model, VanScoy *et al*. reported that fosfomycin activity was best correlated with *f*AUC/MIC (R^2=0.76) against an *E. coli* isolate, with moderate correlation with fC_{max}/MIC (R^2=0.62) and an even poorer relationship between bacterial killing and $fT_{>MIC}$

(R^2=0.42) (274). Results of a study conducted in a hollow-fibre infection model (HFIM) against a single isolate of *E. coli* suggested that *f*AUC/MIC was the dynamic index best linked to resistance suppression (267). In the neutropenic murine thigh infection model, Lepak *et al*. (275) examined the PK/PD activity of fosfomycin against *E. coli*, *K. pneumoniae* and *P. aeruginosa* and demonstrated that the PK/PD index best correlated with activity against these organisms was the AUC/MIC ratio (R^2 = 0.70). Net stasis was observed at 24-h AUC/MIC ratio values of 24, 21, and 15 for *E. coli*, *K.*, *pneumoniae* and *P. aeruginosa*, respectively. In that study dose-fractionation was only conducted on a single strain of *E. coli*, and for *K. pneumoniae* and *P. aeruginosa* only dose escalation was used to estimate target exposure for various magnitudes of bacterial killing with the assumption that AUC/MIC was also applicable to *K. pneumoniae* and *P. aeruginosa* (275).

1.7 Treatment challenges for *P. aeruginosa* infections

1.7.1 Variable patient pharmacokinetics

Effective treatment of *P. aeruginosa* infections in critically ill patients is an ever existing and challenging task for clinicians (61). In ICU, prolonged stay, inappropriate empirical antimicrobial therapy, and repeated and prolonged administration of antibiotics are identified as potential risks for critically ill patients (58-60). However, several other factors associated with pathophysiological changes in these patients have the potential to modify the response to antibiotic therapy. For example, augmented renal clearance can lead to enhanced elimination of many antibiotics resulting in suboptimal antibiotic exposure and diminished antibacterial effect, while impaired renal function can lead to unwanted increased exposure which may precipitate antibiotic-associated toxicity; either of these scenarios may have an adverse impact on the prognosis of the patient (146, 276, 277). Given *P. aeruginosa* can easily mutate and rapidly develop resistance at suboptimal antibiotic exposures, there is increased risk of

therapeutic failure and/or the emergence of resistance (278, 279). Thus, it is essential to recognise the importance of individualising dosage regimens.

In CF patients, the pharmacokinetics of a number of antibiotics including β-lactams, aminoglycosides and fluoroquinolones, may also be altered (280, 281). Patients with CF generally have a larger volume of distribution for many antibiotics, including β-lactam agents and aminoglycosides, due to the lower fat stores in these patients and an increased ratio of lean body mass to total body mass compared with the non-CF population (282). Consequently, larger loading doses of antibacterial agents are required to attain the same serum peak concentration as in non-CF individuals (283). Enhanced total body clearance of antibiotics has also been observed within the CF population. Increased renal clearance, decreased protein binding, and extrarenal elimination, have been proposed as possible reasons for this increased clearance although the exact mechanism for increased elimination is unknown (284).

As increased clearance can result in suboptimal concentrations, which may lead to the emergence of resistance, increased maintenance doses are often required to maintain clinically relevant concentrations. The increased volume of distribution and enhanced clearance of antibiotics, along with the variability of the infection site *(i.e*. ELF or lung fluid) to plasma antibiotic concentration ratio (as discussed in Section 1.5) make antibiotic dosing to achieve therapeutic drug concentrations a real challenge in patients with CF (282). In the case of *P. aeruginosa* isolates with increased MICs, the concentrations achieved at the infection site after intravenous administration may not be sufficient and alternative strategies are required which will be discussed in detail in Section 1.8 of this chapter.

1.7.2. Complexity of infection due to hypermutation and biofilm formation

P. aeruginosa infections in people with CF often require repetitive cycles of lengthy courses of antibiotic therapy (282). However, *P. aeruginosa* infections are typically treated with either nonoptimised monotherapy or empirically chosen nonoptimised combinations, risking the

emergence of MDR hypermutable strains (285). As discussed in Section 1.4, hypermutable *P. aeruginosa* strains are prone to rapidly acquire resistance due to amplification of pre-existing mutants. In a SCTK study, Oliver *et al*. demonstrated that a large proportion of resistant mutants leading to emergence of resistance appeared within 24-36 h of the exposure to all 11 antipseudomonal agents when tested in monotherapy against a hypermutable *P. aeruginosa* strain (PAOΔ*mutS*), as compared to a non-hypermutable *P. aeruginosa* strain (PAO1) (53). That finding was supported in 10-day dynamic HFIM studies in which different meropenem dosing regimens were unable to suppress the emergence of resistance and resulted in high level resistance of hypermutable *P. aeruginosa* strain PAOΔ*mutS* (168). In contrast, the same meropenem monotherapy regimens suppressed bacterial growth to <4 $\log_{10}$ CFU/mL over 7 to 9 days for the non-hypermutator *P. aeruginosa* strain PAO1. The PAOΔ*mutS* strain differs from PAO1 only by the absence of the *mutS* gene. Deletion of *mutS* is one of the most frequent mutations in clinical hypermutable *P. aeruginosa* isolates, and it represents nearly a worst-case scenario, as it has a large impact on increasing mutation rate, hence requiring increased minimum bactericidal concentrations with single agents (meropenem, imipenem, and ceftazidime) to maximise bacterial killing and suppress emergence of resistance (53).Hypermutable *P. aeruginosa* strains are associated with the transition of early (planktonic phase) infection to a respiratory infection involving biofilm formation (52, 286). The biofilm matrix is dominated by alginate and exopolysaccharides that restrict the access of antibiotics to the infecting pathogen and also diminish their antibacterial activity in other ways. Biofilm bacteria become less susceptible to antimicrobials for four main reasons (106):

1. Biofilm bacteria display a different phenotype and become intrinsically less susceptible to most antimicrobials, mainly due to their slow rate of replication. Antibiotics such as β-lactams, fluoroquinolones and aminoglycosides need actively dividing cells for action (287, 288).

2. Antibiotics may not reach their bacterial targets. This may be due to reduced diffusion through the biofilm matrix and/or inactivation of the antibiotic within the matrix. This is the case for β-lactams, which are inactivated by extracellular β-lactamases excreted into the biofilm by bacteria, and for aminoglycosides, which are less active at an acidic pH. Matrix thickness is related to biofilm age (289, 290). Some studies suggested that *P. aeruginosa* cells in young biofilms are much more susceptible to antibiotics than those in more developed (old) biofilms, underlining the importance of early interventions in the treatment of biofilm infections (287, 291).

3. The complementary activity of the immune system is impaired in the biofilms. Adaptive resistant bacterial forms, such as persisters, are usually cleared by macrophages once antibiotics have substantially reduced the bacterial inoculum. However, phagocytic activity of white cells is inhibited by the presence of biofilm (288, 292).

4. Horizontal gene transfer rates are often higher in biofilm communities than those in planktonic states. In addition, biofilms promote plasmid stability and may increase the host range of mobile genetic elements that are transferred horizontally. In this way, biofilm bacteria are able to enhance their resistance to antibiotics (125).

P. aeruginosa has a large armamentarium of resistance mechanisms and its ability to become hypermutable and to form biofilm renders *P. aeruginosa* infections difficult to treat (103). Several *in vitro* and *in vivo* experiments demonstrated that the minimal biofilm inhibitory concentration (MBIC) of many antibiotics is usually more than 1-2 fold dilutions higher than the MIC for planktonic bacteria, complicating the treatment of respiratory exacerbations in CF patients because of difficulty achieving effective concentrations with conventional intravenous dosing schemes (56, 293, 294). Studies described in Chapters 3-5 of this thesis have employed different PK/PD approaches to optimise the administration of antibiotics to maximise the bacterial killing and minimise emergence of resistance. These approaches are discussed in detail in the next section of this chapter.

1.8 Approaches to combat *P. aeruginosa* infections

Designing optimal antibiotic treatment regimens to combat *P. aeruginosa* infections is a major challenge for clinicians and researchers (295, 296). For MDR *P. aeruginosa* infections in critically ill patients, sometimes therapeutic options are limited (297). For patients with CF suffering from exacerbations of *P. aeruginosa* infections, current antibiotic dosing regimens are suboptimal; monotherapy and combination regimens are used empirically in a nonoptimised way (285, 298). The problem is exacerbated by the lack of efficacy of treatments reliant on traditional antipseudomonal antibiotics, which often do not effectively eradicate *P. aeruginosa* and are also being challenged by the development of resistance. Hence, there is a need to design innovative strategies to treat such infections. Subsections 1.8.1-3 provide an overview of approaches investigated in this thesis to combat difficult-to-treat infections caused by *P. aeruginosa* in critically ill or people with CF.

1.8.1 Optimising the use of an 'old' antibiotic fosfomycin by investigating PK/PD indices

The growing threat from 'superbugs' resistant to almost all available antibiotics, including *P. aeruginosa*, is a major medical challenge and has been outlined in Sections 1.2-1.3 (18, 25). However, some 'old' antibiotics, such as fosfomycin, developed decades ago still retain significant activity against a range of MDR Gram-negative bacteria, including *P. aeruginosa* (252, 299). These antibiotics, which were not subjected to contemporary development procedures prior to their approval for clinical use many decades ago, have remained underutilised due to a scarcity of information on their PK, PD and PK/PD; this information is required to design optimal dosage regimens for their optimal use (300, 301). However, over the last 15 to 20 years significant pharmacological research effort on a small number of 'old' antibiotics has brought them back into the clinics; the polymyxins (colistin and polymyxin B) were the first such antibiotics to undergo a redevelopment procedure, which has ultimately

resulted in scientifically informed dosage guidelines for a variety of patient groups, although toxicity with the polymyxins is still common (302-304). Thus, while the polymyxins have temporarily provided another weapon in the clinician's arsenal of currently available antibiotics for treatment of infections caused by *P. aeruginosa* more, and safer, alternatives are still urgently required.

A similar redevelopment process to that undertaken for the polymyxins is required for other 'old' agents such as fosfomycin where PK, PD, PK/PD and other scientific information essential in determining optimal dosage regimens is yet to be determined (305). Several preclinical and clinical studies have shown fosfomycin to be a promising agent, especially as part of combination therapy, for the treatment of various infections caused by both MDR Gram-positive and Gram-negative bacteria, including *P. aeruginosa* (251, 306-310). Given its excellent safety profile, fosfomycin has been suggested as a promising agent for managing infections caused by Gram-negative bacilli resistant to commonly used antibiotics (311). Unfortunately, the development of fosfomycin (first isolated from *Streptomyces* species in 1969) (312) occurred when drug development was conducted more or less on a trial and error basis (313). Consequently, when this project was initiated there was a dearth of knowledge on the PK, PD and PK/PD properties required for the optimal use of fosfomycin (305). This lack of information presented a major limitation for the use of fosfomycin and carried significant risks for patient outcomes, adverse events and resistance emergence, especially for complicated infections such as those caused by *P. aeruginosa* in critically ill patients (300, 314). Hence, it was important to investigate exposure-response relationships (PK/PD indices) for fosfomycin. Defining the relationship between exposure and bacterial killing and emergence of resistance to elucidate the PK/PD index that correlates most closely with activity and then identify PK/PD targets for various magnitudes of activity is crucial in the design of rational dosing strategies for fosfomycin.

In Chapter 2 of this thesis an *in vitro* one-compartment PK/PD model was used to: (i) identify which of the PK/PD indices ($fT_{>MIC}$, $fAUC/MIC$, fC_{max}/MIC) best predicts bacterial killing of fosfomycin against *P. aeruginosa*, and (ii) determine the magnitude of the predictive PK/PD index required to achieve various extents of bacterial killing, and the magnitude of the index needed to prevent the emergence or amplification of fosfomycin-resistant mutants. For this study, one laboratory reference strain and two MDR clinical *P. aeruginosa* isolates were employed, and dose fractionation studies were carried out for in total 35 different dosing regimens across the three strains. Dosing regimens were selected to maximally differentiate among the three PK/PD indices under investigation, and included a wide dose (concentration) range allowing exploration of the complete dose-response relationship from essentially no effect to maximum effect.

Dose fractionation is an essential design aspect of such studies because AUC and C_{max} are linearly related to dose, and simply increasing dose does not allow differentiation among the three PK/PD indices. This can be seen in panel A of Figure 8, reproduced from Craig *et. al.* (131), where a fourfold-higher dose produces a higher C_{max}/MIC, higher AUC/MIC, and a greater $T_{>MIC}$ at constant clearance and half-life. If this higher dose produces an enhanced therapeutic effect, it is not possible to determine which PK/PD index is best predictive of bacterial killing. To overcome this problem, a dose-ranging study that incorporates dose fractionation is needed to reduce the interdependence among the indices (135, 191). Dose fractionation involves dividing the daily dose into various fractions that are administered at appropriate intervals (*e.g.* at a given daily dose, there may be 50% of daily dose administered every 12 h (Q12h), 33.3% of daily dose Q8h, 25% of daily dose Q6h, 16.7% of daily dose Q4h, in addition to the entire daily dose administered Q24h). As shown in panel B of Figure 8, a dose administered every 2 h results in a lower C_{max}/MIC but a longer $T_{>MIC}$ 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 (131).

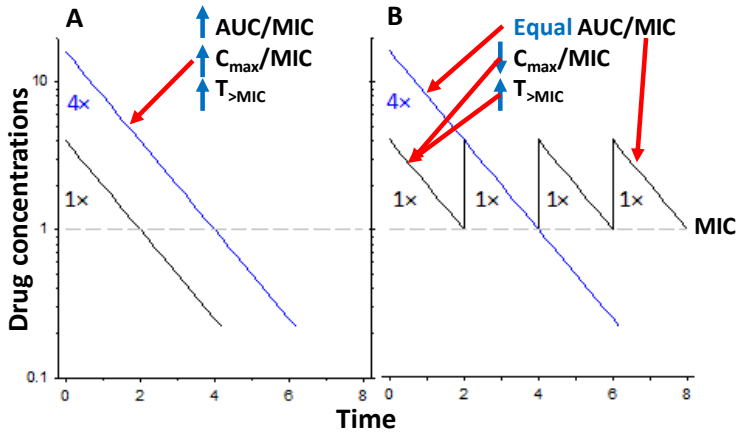

Figure 8: Effect of increasing the dose or changing the dosing regimen of a hypothetical drug on C_{max}/MIC, AUC/MIC and $T_{>MIC}$ (131).

Ultimately, the results obtained from Chapter 2 will provide essential information required for designing optimal dosing regimens of fosfomycin for its rational use in diverse patient populations.

1.8.2 Optimising antibiotic exposure and antibacterial effect

With knowledge of the PK/PD index that correlates most closely with the antibacterial activity of an antibiotic, it is possible to modulate the mode or route of administration to maximise the target exposure to achieve the desired response from a safe daily dose.

1.8.2.1 Modulating the mode of intravenous administration of antibiotics

As discussed in Sections 1.6 and 1.8.1 (and demonstrated in Chapter 2), relevant information about the pattern of bacterial killing by an antibiotic and its PK/PD index is essential to design dosage regimens to maximise the probability of a favourable outcome in patients. *In vitro* and *in vivo* studies have demonstrated that the time-course of antibacterial activity can vary markedly for different antibiotics (131, 145). As discussed in Section 1.6, β-lactam antibiotics have minimal concentration-dependent effect on the rate of bacterial killing and require longer durations for action and generally produce a minimal to moderate PAE (142, 144). Several *in*

vitro and *in vivo* experiments have shown that the $fT_{>MIC}$ should be ≥60-70% and ≥40% of the dosing interval for cephalosporins and carbapenems, respectively, to achieve at least 2 $\log_{10}$ bacterial killing at 24 h (135, 142, 145). On the other hand, some studies have suggested that an even higher $fT_{>MIC}$ is needed for the clinical and microbiological success of meropenem in immunocompromised patients, such as critically ill and neutropenic patients (315-317). For serious bacterial infections, PK/PD targets for meropenem such as 100% $fT_{>4\text{-}5xMIC}$ and an fC_{min}/MIC of ≥4 to 6 have been suggested for resistance suppression and clinical success (318-321). For ceftazidime, a target of >45% $fT_{>MIC}$ was reported as microbiologically and clinically effective to treat patients with nosocomial pneumonia (322).

Thus, for β-lactams the goal of a dosage regimen would be to optimise the duration of exposure (*i.e.* maximise the time for which unbound concentrations exceed the MIC). Although administration of β-lactams by intermittent short-term infusions (*e.g.* 0.5 h) is still the standard mode of administration in many parts of the world (323), this dosing approach does not maximise $fT_{>MIC}$ for a given daily dose and often results in suboptimal antibiotic exposure; as the concentration falls below the MIC, regrowth and emergence of resistance may occur (324, 325). Prolonging the duration of each intermittent infusion (*e.g.* to 3-4 h) or administration as a continuous, constant-rate intravenous infusion can increase $fT_{>MIC}$ and produce a greater antibacterial effect (316, 322, 326, 327). Because the maximum rate of bacterial killing occurs at an unbound ceftazidime concentration ~4× MIC, it was suggested that continuous infusion of ceftazidime to maintain an unbound concentration 4-fold higher than the MIC should maximise efficacy (328, 329). In a retrospective review involving a small number of patients, Prescott *et al.* reported that continuous infusion of ceftazidime appeared to be effective and safe for the treatment of pulmonary exacerbations in CF patients (330). Using previously reported PK data for patients with CF, Thompson *et al.* reported that prolonged and continuous infusion of β-lactams provided higher probability of target attainment than bolus dose regimens (331).

In Chapters 3-5 of this thesis, which focus on antibiotic combinations for treatment of infections caused by hypermutable and biofilm-forming strains of *P. aeruginosa* in patients with CF, we have modulated the exposure ($fT_{>MIC}$) of β-lactams (meropenem, ceftazidime) by altering the mode of administration. In one of those studies, the same daily dose of meropenem was administered as intermittent short-term infusions (0.5 h infusion every 8 h), to mimic a standard regimen, and the antibacterial response was compared with that from administration as a continuous infusion. In the other studies, meropenem or ceftazidime were administered as continuous infusion to maximise the $fT_{>MIC}$.

As discussed in Section 1.6, aminoglycosides and fluoroquinolones exhibit concentration-dependent killing and the most important PK/PD indices correlating with antibacterial activity of each of these antibiotic classes are *f*AUC/MIC and fC_{max}/MIC (135, 142, 146, 191). The *f*AUC/MIC is a time-averaged exposure-response (*i.e.* PK/PD) index and suggests the same extent of bacterial killing regardless of the shape of the concentration-time profile across a day (Figure 8). As noted above, several previous studies demonstrated that for both aminoglycosides and fluoroquinolones fC_{max}/MIC is also important as a determinant of antibacterial effect, indicating that the shape of the concentration-time curve has an impact on the magnitude of antibacterial activity. From results of SCTK studies Rees *et. al.* reported that high ciprofloxacin concentrations over 1-10 h yielded more rapid and extensive initial killing compared to 16 and 24 h exposures with the same *f*AUC/MIC (235). In another SCTK study the same investigators reported that high tobramycin concentrations over 1 and 4 h resulted in more rapid and extensive initial killing compared with 10 and 24 h exposures at the same *f*AUC/MIC (332). With intravenous tobramycin, a high fC_{max}/MIC can be achieved by administering the drug as short-term infusions every 24 hours, a regimen that also minimises the potential of aminoglycoside-associated nephrotoxicity (333, 334). In a HFIM study with ciprofloxacin, Zinner *et al.* reported an extensive emergence of resistance at antibiotic concentrations that fell into the mutant selection window, with a regimen generating a

ciprofloxacin *f*AUC/MIC of 180 administered as 1-h infusion 12 hourly for three days against *P. aeruginosa* strains (335). An earlier dose-fractionation study in the HFIM by Marchbanks *et al.* reported extensive resistance emergence of *P. aeruginosa* isolates with simulated regimens of 400 mg of ciprofloxacin dosed every 8 h and 600 mg every 12 h (equivalent to an *f*AUC/MIC of 60) (336). Resistance emergence at 24 h was less extensive for one isolate and absent for a second *P. aeruginosa* isolate for 1200 mg of ciprofloxacin every 24 h, but for this regimen regrowth of mostly susceptible *P. aeruginosa* at 24 h was observed which was associated with a prolonged period of relatively low ciprofloxacin concentrations over the later stage of the 24-h dosing interval. For the studies involving intravenous regimens described in Chapters 3-5 we simulated exposures of tobramycin and ciprofloxacin as high intensity short-duration intermittent infusions (0.5 h infusion 24 hourly and 1 h infusion 8 hourly, respectively), to attain high, clinically achievable concentrations early in each dosing interval to maximise the antibacterial effects and, in the case of tobramycin, achieve concentration *versus* time profiles expected to lower the risk of nephrotoxicity in patients.

1.8.2.2 Inhalation administration of antibiotics to deliver high concentrations to the site of infection

Typically, intravenous administration of aminoglycosides has been preferred and continues to be used for acute exacerbations of respiratory infections in patients with CF (337-341). However, mechanistic justification and clinical evidence for the efficacy of this route of administration over inhalation remain to be confirmed (342). For serious bacterial infections, an *f*AUC/MIC of >70 and an fC_{max}/MIC of 8-10 have been proposed as PK/PD targets for clinical success in patients receiving intravenous tobramycin (146). These targets are often not achieved for isolates with increased MICs. The use of intravenous regimens to treat respiratory infective *P. aeruginosa* exacerbations in people with CF is especially challenging, due to increased clearance which necessitates administration of aminoglycosides in higher doses than in other patients (218, 343). In addition, following intravenous administration the

penetration of aminoglycosides into lung fluid is less than complete relative to the exposure observed in plasma (221). Hence, there is considerable interest in administration of aminoglycosides *via* inhalation as a possible strategy to combat infective pulmonary exacerbations in CF patients (342). Current treatment guidelines for CF have also endorsed the use of inhaled anti-pseudomonals for patients with *P. aeruginosa* infection, either as an important part of early eradication strategies, or for long-term treatment of chronic *P. aeruginosa* infection, to restore lung function and decrease bacterial load (344).

In fact, this mode of delivery ensures high concentrations of the antibiotics in the infected tissues (lungs) (345-347) leading to greater probability of attainment of PK/PD targets. Data from different studies comparing peak tobramycin concentrations in serum and sputum show that administration of inhalation tobramycin results in low serum concentrations, whereas sputum levels more than 1000-fold greater than those in serum were achieved (345, 348). Thus, administering antibiotics for treatment of lung infections by the inhalation route may be advantageous for antibiotics with narrow therapeutic windows (*e.g*. tobramycin) due to achievement of higher concentrations in lung fluid than can be safely achieved with intravenous administration; for tobramycin this implies reduced risk of nephrotoxicity (349, 350). In a Cochrane review considering the randomised controlled trials in children and adults with CF with early *P. aeruginosa* infection, the authors considered the relative efficacy of antibiotic treatments (combinations of inhaled, oral or intravenous antibiotics *versus* placebo, usual treatment or other combinations of inhaled, oral or intravenous) regarding eradicating the *P. aeruginosa*, delaying the onset of chronic infection, and effects on clinical improvement (351). The quality of the trials was ranked as low to moderate, and the authors concluded that there was insufficient evidence to determine which antibiotic strategy should be used for the eradication of early *P. aeruginosa* infection in CF (351). Another recent Cochrane systematic review considered the randomised controlled trials of inhaled antibiotics for pulmonary exacerbations in patients with CF (342). In that analysis, inhaled antibiotics alone were

compared with intravenous antibiotics alone (2 trials with 77 participants in total), and inhaled antibiotics plus intravenous antibiotics were compared with intravenous antibiotics alone (2 trials with 90 participants in total). Due to the small number of trials and participants, and deficiencies in trial design, the authors of that review were unable to demonstrate whether one treatment was superior to the other or not, and they recommended that further research is needed (342). Thus, in Chapter 5 of this thesis for the first time we systematically investigated the antibacterial effect of clinically relevant lung fluid concentration-time profiles of tobramycin inhalation against hypermutable *P. aeruginosa* isolates. The studies were conducted with simulated concentration-time profiles in an *in vitro* infection model that enabled examination of antibacterial effects on both planktonic and biofilm bacteria.

1.8.3 Enhancing antibacterial effects with combination therapy

In general, monotherapy is still a common practice for the treatment of certain bacterial infections involving susceptible isolates. However as discussed in Section 1.6, *P. aeruginosa* has a large armamentarium of resistance mechanisms and can rapidly become resistant to virtually all currently available antibiotics in monotherapy (34, 116). Several studies have already documented the extensive emergence of resistance with monotherapies used against Gram-negative bacteria, including *P. aeruginosa* (167-169, 204, 332, 352, 353). In a 24-h dynamic study utilising an *in vitro* one-compartment model against non-hypermutable PAO1 and a hypermutable *P. aeruginosa* clinical isolate, a simulated dosing regimen of meropenem 1 g every 8 hours for 24 h with doses administered as short infusions with peak concentration of 56.1 mg/L and half-life of 0.45 h was examined. Extensive emergence of resistance as early as 16 h was observed for the hypermutable isolate, but PAO1 did not show a similar percentage of resistant population until 24 h (354). The use of a one-compartment PK/PD model allowed the investigators to mimic the PK as seen in patients, however the study duration was only 24 h and examined only planktonic bacteria. Studies with longer durations and involving measurement of effects on biofilm bacteria would have allowed further evaluation

of this regimen. For a meropenem-susceptible hypermutable *P. aeruginosa* strain it was demonstrated in a 10-day HFIM study that continuous infusion of meropenem to achieve a concentration as high as ~8× MIC was unable to suppress the emergence of less-susceptible planktonic bacteria (168). In the same study, a simulated dosing regimen of tobramycin 10 mg/kg 24-hourly (*f*AUC/MIC of 217) was also ineffective in suppressing the emergence of resistance despite achieving PK/PD targets (168). Against non-hypermutable *P. aeruginosa,* similar results have been reported with ceftazidime monotherapy in dynamic *in vitro* models simulating clinically achieved serum concentrations resulting from both intermittent and continuous administration (355, 356). In a granulocytopenic murine thigh infection model, it was reported that 23 out of 24 gentamicin-susceptible P. aeruginosa strains were harbouring gentamicin stable resistant subpopulations and were not affected by therapeutic plasma concentrations of gentamicin (366). However, those studies did not quantify the time-course of bacterial killing of biofilm bacteria, nor did they examine the emergence of resistance in biofilm. Importantly, in all studies the antibiotic concentrations examined reflected those achieved in plasma after intravenous administration, but concentrations clinically achievable in lung fluid with this route of administration are generally lower. The use of antibiotic monotherapy against hypermutable *P. aeruginosa* isolates from patients with CF is especially problematic due to the rapid ascent to dominance of less susceptible mutants (98). As a result of potential risk of treatment failure due to selection of resistant mutants and inability to meet PK/PD targets with single agents (53), combination therapies are often suggested to improve efficacy and broaden antimicrobial activity, minimise or eliminate the emergence of resistance, and possibly allow use of lower doses of individual antibiotics thereby minimising adverse effects (357). Indeed, current CF treatment guidelines recommend the use of antipseudomonal agents in combination for the treatment of *P. aeruginosa* exacerbations (339, 340, 341). However, in clinical practice the antibiotics used as a part of combination therapy and the dosing regimens of each antibiotic are often selected empirically, by trial and error or based on personal experience or preference of clinicians. This is not the best approach to achieve a desired clinical outcome.

Numerous studies with non-hypermutable *P. aeruginosa* showed β-lactam plus aminoglycoside combinations to be synergistic by using MIC testing and checkerboard methods (358-360). Unfortunately, these methods do not use clinically relevant 42 dynamic concentrations, and they do not involve quantification of viable cells and suppression of resistance. Several 24-72 h SCTK studies demonstrated that a β-lactam in combination with a fluoroquinolone or an aminoglycoside synergistically kill *P. aeruginosa* (167, 204, 357, 361). Dynamic studies using an *in vitro* one-compartment model also reported combinations as an effective and synergistic option for *P. aeruginosa* infections (169, 362, 363). HFIM studies have also reported the synergistic bacterial killing by a β-lactam with an aminoglycoside or a fluoroquinolone against a range of hypermutable and non-hypermutable *P. aeruginosa* isolates (166, 167). Studies utilising various *in vivo* infection models showed that β-lactam plus aminoglycoside combinations were synergistic against *P. aeruginosa*. Meropenem plus tobramycin was synergistic in a murine pneumonia model, against a double susceptible *P. aeruginosa* isolate (364), and a 24-h neutropenic murine thigh infection model study demonstrated synergistic bacterial killing of a β-lactam plus an aminoglycoside combination against a double resistant *P. aeruginosa* isolate (365). In a granulocytopenic murine thigh infection model, gentamicin combined with ticarcillin was synergistic against a double susceptible (based on MICs) *P. aeruginosa* isolate that harboured an aminoglycoside-resistant subpopulation (367). In another study, the same authors suggested that antibacterial activity of this combination could be improved by administering ticarcillin as a constant infusion (368).

It is important to mention that all the above-mentioned studies focused on planktonic bacteria and none of the studies have optimised and evaluated combination dosage regimens against biofilm bacteria in dynamic infection models. In addition, only one study has simulated lung fluid concentrations for the exploration of antibacterial effects against planktonic bacteria (167). In Chapters 3-5 of this thesis, the dynamic *in vitro* CDC biofilm reactor (CBR) was used. The CBR is a well-accepted, state-of-the-art PK/PD model and has many advantages;

it allows for the simulation of clinically relevant lung fluid concentration-time profiles as seen in patients, and also enables simultaneous examination of antibacterial effects on both planktonic and biofilm bacteria. The studies conducted in Chapters 3, 4 and 5 of this thesis examined regimens of different combinations of antibiotics against hypermutable strains of P. aeruginosa from patients with CF. The antipseudomonal antibiotics in each of the three combinations investigated in this thesis were selected based on the following considerations:

1) The antibiotics in each combination have different mechanisms of action and resistance to present the opportunity for synergy and to avoid cross-resistance.
2) At least one of the antibiotics in each combination has significant ability to penetrate into the biofilms.
3) At least one of the antibiotics has concentration-dependent bacterial killing to achieve an early reduction in bacterial density.
4) For studies involving simulation of lung fluid concentrations following intravenous administration, preference was given to antibiotics that achieve lung fluid concentrations ≥30% of plasma concentrations.

1.9 Summary

The Gram-negative 'superbug' *P. aeruginosa* is responsibe for devastating and life-threatening infections in critically ill and people with CF. The paucity of new antibiotics in the development pipeline and non-optimised use of existing antibiotics has been identified as a significant driver for the emergence of resistance. There is an urgent need to investigate the optimal treatment regimens of existing antibiotics to combat *P. aeruginosa* infections to ensure the prolonged effectiveness of these antibiotics. This thesis applied modern principles of antimicrobial PK and PD to the 'old' antibiotic fosfomycin to generate, from studies in a one-compartment infection model, essential pre-clinical information needed for the rational design of optimal intravenous dosing schemes for *P. aeruginosa* associated infections in critically ill patients. Given the resilience of hypermutable biofilm-forming *P. aeruginosa* CF isolates and the inability of antibiotic monotherapy to effectively kill and suppress their regrowth and resistance, this thesis also examined combinations of antipseudomonal antibiotics against such isolates which represent a near worst-case scenario. Those studies were conducted in the CBR infection model as it allowed for simulation of clinically relevant lung fluid concentration *versus* time profiles and also enabled examination of the time-course of bacterial killing and emergence or suppression of resistance for both planktonic and biofilm bacteria simultaneously.

2.0 Statement of hypotheses and aims

2.0.1 Hypotheses

The Hypotheses tested in this thesis were that:

1. The PK/PD index of fosfomycin which best predicts bacterial killing and prevention of emergence of resistance of *P. aeruginosa* is the cumulative percentage of a 24-h period that unbound plasma concentrations exceed the MIC [*i.e.* $fT_{>MIC}$].
2. Against both planktonic and biofilm-embedded MDR hypermutable *P. aeruginosa* at simulated lung fluid concentrations, continuous infusion of meropenem in combination with tobramycin provides enhanced bacterial killing and suppression of resistant mutants compared to combinations containing standard intermittent short-term infusions of meropenem.
3. A combination regimen of the fluoroquinolone ciprofloxacin, with meropenem can overcome the emergence of ciprofloxacin-resistant subpopulations and achieve synergistic effects against hypermutable *P. aeruginosa* clinical and reference strains in the planktonic and biofilm state, with simulated lung fluid concentrations of each antibiotic.
4. Continuous infusion of ceftazidime in combination with inhaled tobramycin provides enhanced bacterial killing and suppression of resistant subpopulations compared to combinations containing tobramycin administered intravenously, against both planktonic and biofilm-embedded hypermutable *P. aeruginosa* isolates.

2.0.2 Specific aims to address the hypotheses

To address these hypotheses, the aims were to:

1. Identify the PK/PD index (*i.e.* $fT_{>MIC}$, fAUC/MIC, or fC_{max}/MIC) that best predicts fosfomycin efficacy, and quantify the magnitude of the predictive PK/PD index required to achieve

various extents of bacterial killing or needed to prevent the emergence or amplification of fosfomycin-resistant subpopulations.

2. Characterise the effect of simulated lung fluid concentration-time profiles of meropenem (administered intravenously as either continuous infusion or intermittent short-term infusion) and intravenous tobramycin, in monotherapy and combination, on bacterial killing and resistance emergence against a carbapenem-resistant MDR hypermutable *P. aeruginosa* clinical isolate, in planktonic and biofilm growth.
3. Evaluate intravenous ciprofloxacin and meropenem in monotherapy and combinations at simulated lung fluid concentrations (for different levels of penetration of meropenem) against planktonic and biofilm bacteria for a hypermutable laboratory reference strain and a clinical isolate from a CF patient.
4. Investigate the effect of lung fluid concentration-time profiles of tobramycin (administered as either intermittent intravenous infusions or by inhalation) and ceftazidime as continuous infusion, in monotherapy and combination, on bacterial killing and resistance emergence of hypermutable *P. aeruginosa* clinical isolates, in planktonic and biofilm growth.

2.1 Structure of this thesis

As the methods employed for the studies included in this thesis are described in detail in each chapter, a separate methods chapter has not been included. Aim 1 was addressed by the studies described in Chapter 2, while Aims 2, 3 and 4 were addressed by the studies described in Chapters 3, 4 and 5, respectively. Chapters 2 to 4, (original research findings) comprise manuscripts which have been published. A further original research manuscript is going to be submitted based on the data in Chapter 5. Lastly, all major findings and conclusions from the research detailed in these chapters along with potential future directions are summarised in Chapter 6.

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Chapter 2

Elucidation of the pharmacokinetic/pharmacodynamic determinants of fosfomycin activity against *Pseudomonas aeruginosa* using a dynamic *in vitro* model

Preamble

The use of fosfomycin against MDR *P. aeruginosa* has increased in the past few years, however scarcity of information about its PK and PD properties has limited understanding of how it may be used most effectively. Information on the PK/PD index most closely correlated with fosfomycin activity is important for the design of optimised dosing regimens. The study carried out in this chapter enabled identification of the most predictive PK/PD index driving fosfomycin activity against *P. aeruginosa*. In addressing **Aim 1**, this is the first study to use a dose-ranging and dose-fractionation design in a dynamic one-compartment *in vitro* PK/PD model over 24 h to identify the fosfomycin PK/PD index ($fT_{>MIC}$, *f*AUC/MIC or fC_{max}/MIC) most closely correlated with antibacterial activity, and determine the target values of that index associated with various extents of bacterial killing and the prevention of emergence of resistance. A large range of fosfomycin concentrations (fC_{max} range: 6.25 - 3000 mg/L) and concentration-time profiles were simulated by use of 30 different regimens comprising intermittent dosing (with 8, 12 and 24 h intervals) and constant concentrations. This enabled maximal differentiation among the PK/PD indices under investigation. One reference *P. aeruginosa* strain and two MDR clinical isolates were examined. The findings in this Chapter did not support **Hypothesis 1** that the PK/PD index of fosfomycin which best predicts bacterial killing and prevention of emergence of resistance of *P. aeruginosa* is the $fT_{>MIC}$. The results of this Chapter have been published in the ***Journal of Antimicrobial Chemotherapy***, as such they are presented in the format of a published article in the section immediately following.

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Elucidation of the pharmacokinetic/pharmacodynamic determinants of fosfomycin activity against *Pseudomonas aeruginosa* using a dynamic *in vitro* model

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Objectives: To identify the fosfomycin pharmacokinetic (PK)/pharmacodynamic (PD) index ($fT_{>MIC}$, *f*AUC/MIC or fC_{max}/MIC) most closely correlated with activity against *Pseudomonas aeruginosa* and determine the PK/PD target associated with various extents of bacterial killing and the prevention of emergence of resistance.

Methods: Dose fractionation was conducted over 24 h in a dynamic one-compartment *in vitro* PK/PD model utilizing *P. aeruginosa* ATCC 27853 and two MDR clinical isolates (CR 1005 and CW 7). In total, 35 different dosing regimens were examined across the three strains. Microbiological response was examined by log changes and population analysis profiles. A Hill-type E_{max} model was fitted to the killing effect data (expressed as the $\log_{10}$ ratio of the area under the cfu/mL curve for treated regimens versus controls).

Results: Bacterial killing of no more than ~3 $\log_{10}$ cfu/mL was achieved irrespective of regimen. The *f*AUC/MIC was the PK/PD index most closely correlated with efficacy ($R^2 = 0.80$). The *f*AUC/MIC targets required to achieve 1 and 2 $\log_{10}$ reductions in the area under the cfu/mL curve relative to growth control were 489 and 1024, respectively. No regimen was able to suppress the emergence of resistance, and near-complete replacement of susceptible with resistant subpopulations occurred with virtually all regimens.

Conclusions: Bacterial killing for fosfomycin against *P. aeruginosa* was most closely associated with the *f*AUC/MIC. Suppression of fosfomycin-resistant subpopulations could not be achieved even with fosfomycin exposures well above those that can be safely achieved clinically.

Introduction

Effective treatment of infections caused by MDR Gram-negative pathogens such as *Pseudomonas aeruginosa* is a major medical challenge.[1–3] *P. aeruginosa*, previously identified by the IDSA as one of the top six pathogens threatening healthcare systems,[4,5] has now been categorized as a 'Serious' threat level by the US CDC.[6] With numerous intrinsic and acquired resistance mechanisms present in this organism,[7] antibiotic resistance across all *P. aeruginosa* infections emerges during therapy in up to 25% of cases and is associated with treatment failure in 50%–85% of patients and greater risk of mortality.[8,9] With a shortage of new antibiotics with novel mechanisms of action in the drug discovery and development pipeline,[10] there is a growing need to optimize the use of older 'forgotten' antibiotics[11] to treat infections, including those caused by *P. aeruginosa*.[12]

Fosfomycin is an older antibiotic exhibiting activity against many Gram-negative pathogens, including a significant subset of MDR *P. aeruginosa* strains.[13–15] Given that it is generally well tolerated,[16] fosfomycin has been suggested as a promising agent for managing infections caused by Gram-negative bacilli that are resistant to commonly used antibiotics.[17] Unfortunately the development of fosfomycin (first isolated from *Streptomyces* species in 1969)[18] occurred when drug development was conducted more or less on a trial-and-error basis.[19] Consequently, there is a dearth of knowledge on the pharmacokinetic (PK) and pharmacodynamic (PD) properties of fosfomycin required to optimize therapy.[20] This lack of established regimens specifically for complicated infections

is a primary limitation to the use of fosfomycin and carries significant risks for patient outcomes, adverse events and resistance emergence.[13,21]

It has been recommended that exposure–response relationships for older antimicrobials, including fosfomycin, be urgently established.[17,20] The determination of the relationship between bacterial killing and emergence of resistance with respect to PK/PD indices and the determination of PK/PD targets will assist in the design of rational dosing strategies for fosfomycin. Therefore, we utilized an *in vitro* PK/PD model (i) to identify the PK/PD index [i.e. the cumulative percentage of a 24 h period for which unbound concentrations exceed the MIC ($fT_{>MIC}$), the area under the unbound concentration–time curve to MIC ratio (*f*AUC/MIC) or the unbound maximal concentration to MIC ratio (fC_{max}/MIC)] that best predicts bacterial killing of fosfomycin against *P. aeruginosa*; and (ii) to determine the magnitude of the predictive PK/PD index required to achieve various extents of bacterial killing and/or prevent the emergence or amplification of fosfomycin-resistant mutants.

Materials and methods

Antibiotics, bacterial isolates and MIC testing

Fosfomycin disodium (Lot 20131012, Waterstone Technology, Carmel, IN, USA) and glucose-6-phosphate (G6P; Lot SLBD7775V, Sigma-Aldrich, Castle Hill, NSW, Australia) were supplied by their respective manufacturers. Sterile stock solutions were prepared in Milli-Q water immediately prior to each experiment. Cation-adjusted Mueller–Hinton agar and CAMHB supplemented with 25 mg/L G6P per CLSI guidelines were used in all experiments.[22]

Three fosfomycin-susceptible strains of *P. aeruginosa* were examined: reference strain ATCC 27853 (ATCC, Manassas, VA, USA) and two previously described MDR clinical isolates [CR 1005 (non-mucoid) and CW 7 (mucoid)].[23] MDR was defined as non-susceptibility to at least one antimicrobial agent in three or more antimicrobial categories.[24] The MICs, determined in duplicate on separate days using agar dilution per CLSI guidelines,[22] were 8 mg/L for ATCC 27853, 32 mg/L for CR 1005 and 16 mg/L for CW 7. As breakpoints for fosfomycin against *Pseudomonas* spp. are currently lacking, we applied modified CLSI breakpoints for *Escherichia coli* with an MIC $\leq$64 mg/L considered susceptible and >64 mg/L resistant.[22]

Population analysis profiles

The possible presence of fosfomycin-resistant subpopulations within the predominant (susceptible) population at baseline was determined via population analysis profiles (PAPs) (inoculum $\sim10^8$ cfu/mL) for each strain as described previously.[23] Fosfomycin heteroresistance was defined as the presence within a fosfomycin-susceptible isolate (i.e. MIC $\leq$64 mg/L) of subpopulations able to grow on agar containing >64 mg/L fosfomycin. Random colonies were selected from fosfomycin-containing agar plates for repeated MIC testing to confirm the increased MICs.

Dynamic *in vitro* PK/PD model, fosfomycin dosing regimens and emergence of resistance

A previously described dynamic *in vitro* PK/PD model[25] was used over 24 h to examine the PK/PD index that best predicts the antimicrobial response of fosfomycin. Prior to each experiment, strains were subcultured onto Mueller–Hinton agar (Media Preparation Unit) and incubated overnight at 35°C. One colony was then selected and grown overnight in 10 mL of CAMHB from which early log-phase growth was obtained. A 1 mL aliquot was then injected into each central compartment to yield a starting inoculum of $\sim10^6$ cfu/mL.

Both continuous infusion and intermittent dosing regimens were simulated as described previously,[25] with serial samples for viable cell counting and determination of fosfomycin concentrations collected aseptically as shown in Table 1. For intermittent regimens an elimination half-life ($t_{1/2}$) of 4 h was simulated, approximating fosfomycin elimination in critically ill patients[26,27] and healthy volunteers.[28,29] Given that fosfomycin has negligible plasma protein binding,[30,31] concentrations were assumed to constitute unbound fosfomycin. Viability counting was undertaken as previously described[23] and antibiotic carryover minimized by centrifuging all samples for 5 min at 10000 rpm with resuspension in prewarmed saline (37°C). To additionally examine the presence of fosfomycin-resistant subpopulations at baseline (0 h) and following 24 h of treatment, PAPs were conducted on all isolates for a subset of experiments at these times on Mueller–Hinton agar containing G6P (25 mg/L) and fosfomycin at 32, 64, 128 and 256 mg/L.

Three intermittent dosing intervals (8, 12 and 24 h) with fC_{max} varied across each schedule plus constant concentration (CC) regimens were examined (Table 1). Dosing regimens were selected to maximally differentiate among the PK/PD indices under investigation (*f*AUC/MIC, fC_{max}/MIC and $fT_{>MIC}$) and included a wide concentration range to allow exploration of the complete dose–response relationship from essentially no effect to maximum effect. Fosfomycin concentrations were determined using a previously published LC-MS/MS assay with minor modification.[32] The assay range was 1–500 mg/L; samples were diluted if the expected fosfomycin concentrations were higher than the upper limit of quantification.

Investigation of PK/PD indices

For each dosing regimen the %$fT_{>MIC}$, *f*AUC/MIC, fC_{max}/MIC and the area under the killing curve (AUC_{cfu}) of the time-course profile of bacterial numbers (cfu/mL from 0 to 24 h) were determined as described previously at both 1× and 10× MIC.[25]

The log ratio area method, which mostly compensates for the bacterial loss from the model,[33] was used to quantify the killing effect (drug effect) chosen as the measure of efficacy (E) per the equation: $E = \log_{10}[AUC_{cfu(treatment)}/AUC_{cfu(growth\ control)}]$.

The relationship between killing effect (*E*) and each PK/PD index was analysed as described previously using a Hill equation with a baseline and an inhibitory effect, with the magnitude of the most predictive PK/PD index required to achieve 1 or 2 $\log_{10}$ reduction in the area under the cfu/mL curve relative to growth control estimated from the E_0, E_{max}, EI_{50} and γ.[25]

Results

Baseline PAPs

Baseline PAPs are shown in Figure 1. Despite all strains being considered fosfomycin susceptible based on MICs (MICs of 8, 16 and 32 mg/L), growth occurred on all PAP plates up to and including 256 mg/L. Colonies obtained from plates containing fosfomycin at 128 and 256 mg/L had elevated MICs ($\geq$128 mg/L for ATCC 27853 and $\geq$256 mg/L for CR 1005 and CW 7), indicating that resistant subpopulations were present in all strains prior to treatment. The proportion of bacterial colonies growing on plates containing fosfomycin at 128 mg/L were 4.20×10^{-6}, 1.87×10^{-5} and 2.57×10^{-6} for ATCC 27853, CR 1005 and CW 7, respectively; a similar proportion of subpopulations grew in the presence of 256 mg/L.

Quantification of fosfomycin concentrations, bacterial killing and emergence of resistance

Observed fosfomycin concentrations were on average within 15% of those targeted. Typical profiles showing the relationship between

Table 1. Fosfomycin dosing regimens and sampling times in the *in vitro* PK/PD model[a,b]

	Dosing regimen			
	8 h	12 h	24 h	CC
Target fC_{max} (mg/L)				
ATCC 27853	250, 125, 75, 50, 12.5, 6.25	2500, 1500, 1125, 1000, 750, 425, 250, 63, 32	3000, 2000, 1300, 1000[e], 750, 500[e], 250[e], 125[e], 63[e], 16[e]	50, 25
CR 1005	250, 125, 75, 50, 25, 12.5	750, 500, 63, 32, 16, 8	3000, 2500, 2000, 1500, 750, 500, 250, 63, 32	500, 250, 50, 25
CW 7	250, 125, 75, 50, 12.5, 6.25	500, 63, 32, 16	3000, 2500, 2000, 1500, 500, 250, 63, 32	500, 250, 50, 25
Sampling times (h) for				
microbiological measurements[c]	0, 1, 3, 5, 8, 16, 24	0, 1, 3, 5, 8, 12, 24	0, 1, 3, 5, 8, 24	0, 1, 3, 5, 8, 24
fosfomycin quantification[c,d]	0, 4, 8, 9, 12, 13, 16, 17, 24	0, 4, 8, 12, 13, 24	0, 4, 8, 24	0, 4, 8, 24

[a]Dosing regimens involved intermittent administration at 8, 12 or 24 h to achieve the target steady-state fC_{max} or CC simulating continuous infusion.
[b]Fosfomycin MICs were 8 mg/L for ATCC 27853, 32 mg/L for CR 1005 and 16 mg/L for CW 7.
[c]Initial experiments with multiple-dose regimens (dosing every 8 and 12 h) at high concentrations showed no further bacterial killing at later time-points (12 and 16 h). Consequently, for subsequent experiments sampling was conducted up to 8 h and then at 24 h.
[d]A subset of each dosing regimen (8 h, 12 h, 24 h and CC) was assayed to determine fosfomycin concentrations.
[e]Results from a previous study.[23]

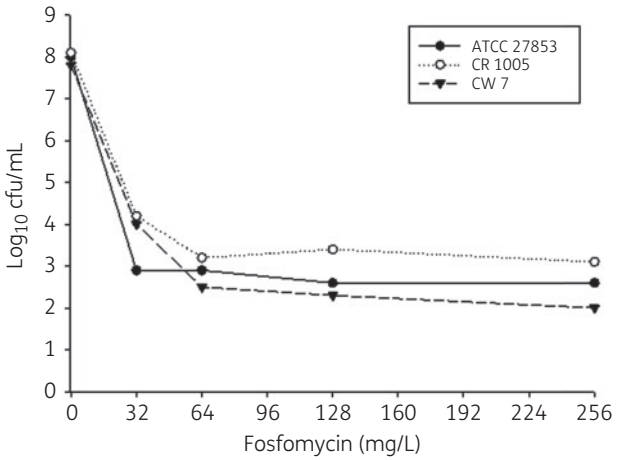

Figure 1. Baseline PAPs of reference strain ATCC 27853 and two clinical isolates. All strains were considered susceptible based on MIC determinations (MICs of 8, 32 and 16 mg/L).

targeted and observed concentrations are shown in Figure 2. Representative time-course profiles of bacterial numbers for each isolate, including the PAPs, are presented in Figure 3. The initial inocula in the control and treatment compartments (mean ± SD) were: 6.03 ($n = 2$) and 5.91±0.24 ($n = 27$) $\log_{10}$ cfu/mL for ATCC 27853, 6.07±0.30 ($n = 3$) and 6.34±0.18 ($n = 25$) $\log_{10}$ cfu/mL for CR 1005, and 6.35±0.12 ($n = 4$) and 6.01±0.15 ($n = 30$) $\log_{10}$ cfu/mL for CW 7. After 24 h, bacterial numbers in the control compartments had increased to 8.1 ($n = 2$), 8.15±0.26 ($n = 3$) and 8.30±0.27 ($n = 4$) $\log_{10}$ cfu/mL for ATCC 27853, CR 1005 and CW 7, respectively.

The rate of initial bacterial killing against each isolate generally increased with increasing fosfomycin concentrations up to ∼10× MIC; further increases did not produce more rapid or extensive killing. With the majority of fosfomycin regimens, initial killing of no more than ∼3 $\log_{10}$ cfu/mL occurred across the first 4–8 h for all isolates followed by regrowth close to control values at 24 h. In a small number of cases with very high-dose regimens [e.g. fC_{max} of 750 mg/L and 1500 mg/L every 12 h against ATCC 27853 (Figure 3, left-hand panel)], regrowth remained below the initial inoculum at 24 h. Maximum bacterial killing achieved against ATCC 27853 was 3.0 $\log_{10}$ cfu/mL using an fC_{max} of 1500 mg/L administered every 12 h. The equivalent values and regimens for CR 1005 and CW 7 were 3.1 $\log_{10}$ cfu/mL with an fC_{max} of 750 mg/L every 12 h and 3.2 $\log_{10}$ cfu/mL with two regimens, fC_{max} of 1500 mg/L every 12 h and 500 mg/L as a CC, respectively.

For the subset of experiments that included PAPs, no fosfomycin-resistant colonies for any strain were detected immediately prior to the commencement of therapy at the starting inoculum of $\sim 10^6$ cfu/mL (Figure 3, right-hand panels). For the growth controls, PAPs following 24 h of incubation indicated the presence of resistant subpopulations in all three strains; growth at this time was $\sim 10^{8-8.5}$ cfu/mL. The proportion of resistant colonies growing on plates containing fosfomycin at 128 mg/L at 24 h was 1.87×10^{-6}, 1.02×10^{-4} and 1.81×10^{-6} for ATCC 27853, CR 1005 and CW 7, respectively. These proportions were similar to those observed with the baseline PAPs (inoculum $\sim 10^8$ cfu/mL). With the exception of the fC_{max} 32 mg/L 12 h regimen against CW 7 (in which the proportion of resistant colonies at 24 h was 3.33×10^{-4}), with all other regimens in which PAPs were performed virtually the entire population at 24 h grew in the presence of fosfomycin at 256 mg/L (Figure 3, right-hand panels).

Relationships between bacterial killing and PK/PD indices

The relationships between killing effect and *f*AUC/MIC, fC_{max}/MIC and $fT_{>MIC}$ are shown in Figure 4. At 1× and 10 × MIC the PK/PD index that best predicted efficacy was *f*AUC/MIC ($R^2 = 0.80$; Figure 4, top row). A poorer relationship existed between the

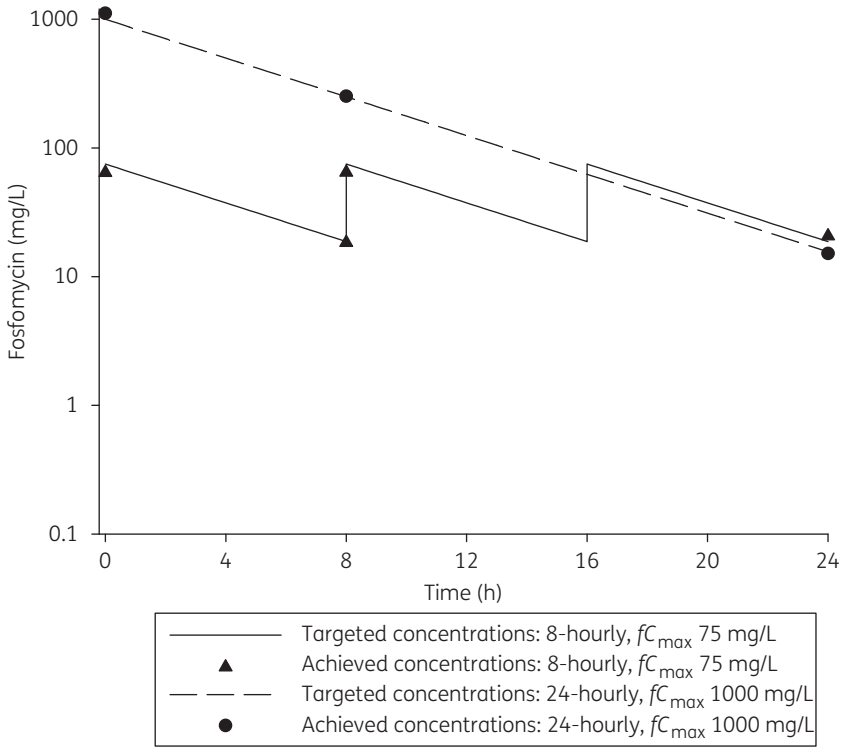

Figure 2. Typical simulated PK profiles showing the relationship between targeted and achieved fosfomycin concentrations in the PK/PD model.

killing effect and fC_{max}/MIC, for which greater scatter and systemic deviations from the curve fit were observed ($R^2 = 0.71$; Figure 4, middle row). No relationship was observed for $fT_{>MIC}$ at 1× MIC (Figure 4c), but one was found at 10 × MIC ($R^2 = 0.70$; Figure 4f). The magnitudes of the *f*AUC/MIC indexes required for 1 and 2 $\log_{10}$ reduction in the area under the cfu/mL curve relative to growth control for each strain were 489 and 1024, respectively.

Discussion

Oral fosfomycin (fosfomycin tromethamine) is currently indicated for treatment of uncomplicated urinary tract infections caused by *E. coli* and *Enterococcus faecalis* in women.[34] However, the parenteral (disodium) formulation is increasingly used to treat systemic infections caused by MDR organisms, including *P. aeruginosa*.[35–37] Unfortunately, the information required to optimize dosing regimens using exposure–response relationships is not available. It has therefore been suggested that establishing the exposure–response relationships for fosfomycin for both efficacy and resistance selection—which are often distinctly different—be made a priority.[20] We sought to determine the predictive performance of potential PK/PD indices with respect to bacterial killing and the emergence of resistance against *P. aeruginosa*, including MDR isolates.

We have previously reported maximal bacterial killing of ∼3 $\log_{10}$ cfu/mL followed by rapid regrowth against *P. aeruginosa* ATCC 27853 in a 24 h PK/PD model using once-daily dosing (fC_{max}, 1000 mg/L).[23] Similar maximal killing was recently shown for the same strain using static time–kill studies (C_{max}, 128 mg/L) and a tissue-cage infection model.[38] This maximal level of killing is almost identical to that achieved here against the same reference strain and two additional MDR clinical isolates with dosing regimens providing a much greater exposure to fosfomycin (e.g. fC_{max} 1000 mg/L every 12 h or fC_{max} 3000 mg/L every 24 h). Importantly, near-complete replacement of susceptible with resistant subpopulations occurred with virtually all regimens. Although fosfomycin-resistant colonies were not detected immediately prior to commencement of therapy in the dose-fractionation studies (inoculum ∼10^6 cfu/mL), ∼2–3 $\log_{10}$ cfu/mL of resistant colonies growing on agar containing 256 mg/L fosfomycin were present in the baseline PAPs (inoculum ∼10^8 cfu/mL). Thus, it is highly likely that resistant subpopulations were present at the commencement of therapy in the dose-fractionation studies but were simply not detected at the lower inoculum. The rapid regrowth observed following commencement of fosfomycin administration was driven, at least in part, by amplification of these pre-existing, highly fosfomycin-resistant subpopulations. This is similar to what we observed previously against *P. aeruginosa* at much lower fosfomycin exposures [maximum $AUC_{0–24}$ of 5680, 2840 mg·h/L (fC_{max} of 1000 and 500 mg/L administered every 24 h)].[23] However, even an $AUC_{0–24}$ of 25247 mg·h/L (fC_{max} of 2500 mg/L administered every 12 h) in the present study could not suppress the emergence of resistance; this is much greater than achieved in patients with standard dosing regimens (typically 4–8 g intravenously every 8 h for serious systemic infections) in which fC_{max} typically ranges from 400 to 1000 mg/L.[26,31,32,39]

Very few studies have previously examined the emergence of fosfomycin resistance. Using a hollow-fibre infection model (half-life, 4 h; initial inoculum, ∼1×10^6 cfu/mL) and a single clinical isolate of *E. coli*, Docobo-Pérez *et al.*[40] simulated human-like

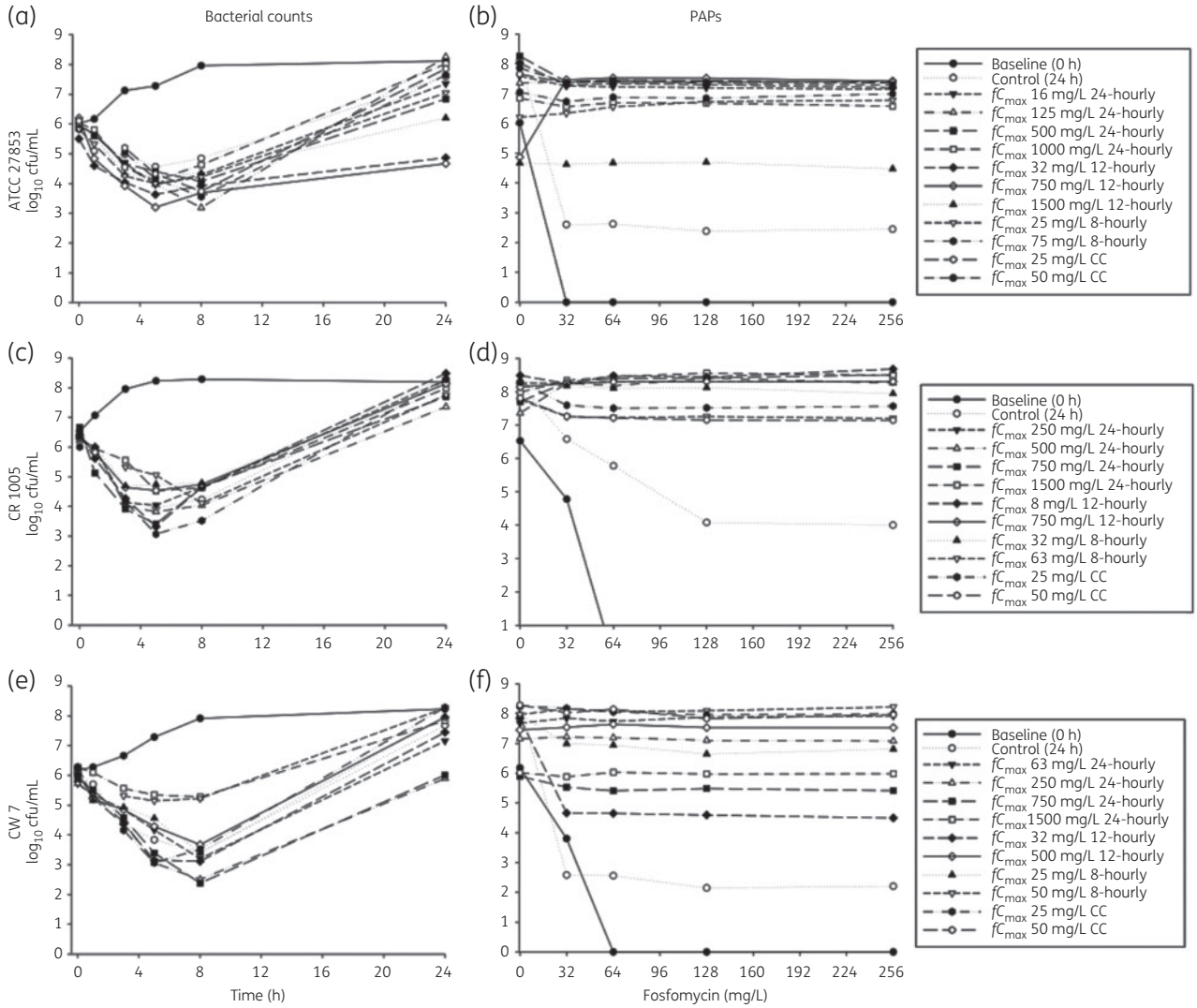

Figure 3. Left-hand panels: representative microbiological responses observed in the *in vitro* PK/PD model simulating the fosfomycin PK of different dosing regimens using ATCC 27853, CR 1005 and CW 7. Right-hand panels: PAPs at baseline (0 h) and after 24 h of exposure to fosfomycin at an initial inoculum of $\sim 10^6$ cfu/mL.

concentration–time profiles corresponding to fosfomycin regimens of 4, 5, 6 and 8 g every 8 h, with each dose infused over 1 h, or 24 g every 24 h administered as a single bolus. For the two 24 g/day regimens (administered once daily or as divided doses) no viable bacteria were detected at 40 h. For all other regimens, rapid regrowth with amplification of fosfomycin-resistant subpopulations occurred such that by ~64 h the entire population grew on agar containing fosfomycin at 256 mg/L. These results indicate that at least for this single isolate of *E. coli* resistance suppression is achievable. Given that both the 8 g/8 h and 24 g/24 h regimens eradicated the entire population, the authors concluded that the *f*AUC/MIC was the dynamic index best linked to resistance suppression. VanScoy *et al.*[41] were similarly able to suppress bacterial regrowth and the emergence of fosfomycin-resistant subpopulations of a heteroresistant *E. coli* reference strain with regimens containing ≥1 g of fosfomycin administered every 6 h. However, even with these dosing regimens the total population was not eradicated. With regimens containing ≤0.5 g administered every 6 h, rapid bacterial regrowth with amplification of fosfomycin-resistant subpopulations occurred such that the latter completely replaced the total population. They obtained similar results against the same strain in dose-fractionation studies (discussed below) in which emergence of resistant subpopulations, often completely dominating the total population, occurred with lower-dose regimens but was suppressed or even eliminated completely with higher-dose regimens. The relationship between fosfomycin dose and the emergence of resistant subpopulations matched an inverted-U-shaped function, described previously with other antibiotics, including against *P. aeruginosa*.[42,43]

Using single reference strains of *E. coli* and *P. aeruginosa* (ATCC 27853), Pan *et al.*[38] reported selective enrichment of resistant subpopulations (growing on agar containing fosfomycin at 4, 8 and 16× MIC) for both isolates across a specific concentration range, but no development of fosfomycin resistance at a higher

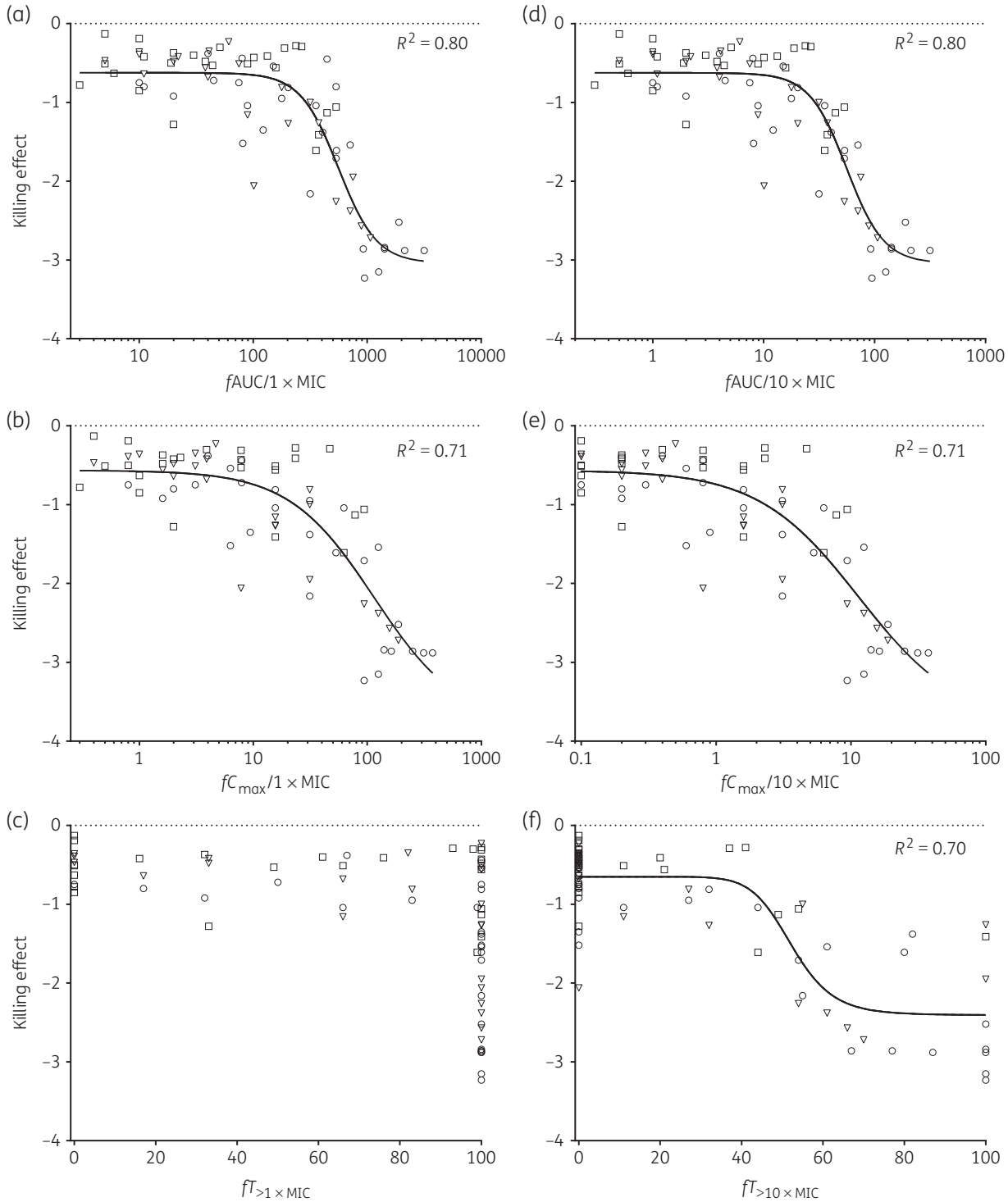

Figure 4. Relationship between the killing effect of fosfomycin against *P. aeruginosa* ATCC 27853 (open circles), CR 1005 (open squares) and CW 7 (open triangles) as a function of three PK/PD indices (calculated at 1× and 10× MIC): *f*AUC/MIC, fC_{max}/MIC and $fT_{>MIC}$. Each data point represents the result from a single treatment run in the dynamic *in vitro* PK/PD model.

concentration. For *P. aeruginosa*, enrichment occurred with concentrations of 16–64 mg/L for time–kill studies, with resistance suppression occurring at 128 mg/L; a similar result was achieved with *in vivo* tissue-cage experiments (24 h dosing with a maximum achieved concentration of ∼300 mg/L). To the best of our knowledge this is the only report indicating suppression of resistance against *P. aeruginosa* with fosfomycin monotherapy. This is an interesting and unexplained result given the extremely high initial

inoculum ($\sim10^9$ cfu/mL). We have previously shown in static time–kill studies a pronounced inoculum effect for fosfomycin against three strains of *P. aeruginosa*, including ATCC 27853, with bacterial killing essentially eliminated at an inoculum of $\sim10^8$ cfu/mL across a concentration range of 1–1024 mg/L; highly resistant subpopulations were enriched with all but the lowest concentrations used.[23] Given our previous observations of a near-complete lack of activity with monotherapy at a high inoculum, we chose a lower inoculum ($\sim10^6$ cfu/mL) for the present study. However, extremely high exposures in the present study (up to an fC_{max} of 3000 mg/L) with a lower starting inoculum were similarly unable to suppress amplification of resistant subpopulations, even with the loss of some bacteria from the system; bacterial loss is a limitation of one-compartment models. This situation would only be made worse if a shorter half-life, which would reduce bacterial killing even further, were to be used; half-lives of 2–3 h have been reported in some critically ill patients.[44,45] Regardless of resistance emergence, absolute bacterial numbers in the study by Pan *et al.*[38] never dropped below $\sim$5–6 $\log_{10}$ cfu/mL. That study notwithstanding, although increasing fosfomycin exposures may prevent or limit the emergence of resistance against other organisms such as *E. coli*, such a relationship does not appear to exist against *P. aeruginosa*. Indeed, we previously found that all of the 14 *P. aeruginosa* isolates examined (MIC range: 1–64 mg/L) contained resistant subpopulations,[23] and that only moderate bacterial killing is achievable even with supra-therapeutic exposures (maximum killing of ~3 $\log_{10}$ cfu/mL). Therefore, against *P. aeruginosa*, treatment failure with fosfomycin monotherapy would appear likely even considering the potential added effect of the immune system,[46] with monotherapy regimens serving only to amplify pre-existing and highly resistant subpopulations. This situation is compounded by the fact that resistance to fosfomycin in *P. aeruginosa* appears to come with no apparent fitness cost.[14,47,48] The difference in resistance suppression between *P. aeruginosa* and *E. coli* may be due to differences in the transport systems required for fosfomycin entry into the cells. *E. coli* contains two transport systems, the glycerol-3-phosphate (GlpT) and a hexose phosphate (UhpT), whereas *P. aeruginosa* contains only GlpT.[49,50] Thus only a single mutation is required in *P. aeruginosa* to prevent fosfomycin entry and render the organism resistant, whereas in *E. coli* two mutations would be required.[47,48,51–53]

Only two previous studies have purported to examine the exposure–response relationships for bacterial killing of fosfomycin against any organisms. VanScoy *et al.*[41] used a one-compartment PK/PD model similar to ours to examine bacterial killing and resistance emergence against one reference strain and two clinical isolates of *E. coli*. Experiments were conducted over 24 h (starting inoculum $\sim1\times10^6$ cfu/mL) and simulated a fosfomycin half-life of 2 h. Three regimens with intermittent administration every 6, 8 and 12 h, as well as a continuous-infusion regimen, were examined, with each providing the same total daily fosfomycin exposure (as measured by the AUC_{0-24}). The authors concluded that fosfomycin activity was most likely linked to *f*AUC/MIC ($R^2 = 0.76$), although there was also a strong correlation with fC_{max}/MIC ($R^2 = 0.62$); there was a poor relationship between bacterial killing and $fT_{>MIC}$ ($R^2 = 0.42$). However, it was noted that given the majority of $fT_{>MIC}$ values were 100%, the PK/PD relationship based on this index could not be adequately explored. Lepak *et al.*[54] used the neutropenic murine thigh infection model to examine the PK/PD activity of fosfomycin against five strains of *E. coli*, three of *Klebsiella pneumoniae* and two of *P. aeruginosa*. Although they determined that the PK/PD index best correlated with activity against these organisms was the AUC/MIC ($R^2 = 0.70$; protein binding was not stated), dose-fractionation was only conducted on a single strain of *E. coli*; for *K. pneumoniae* and *P. aeruginosa*, only increasing doses administered every 3 h were used, with the index determined for *E. coli* assumed to also apply to these organisms. Our dose-fractionation study employed the largest range of fosfomycin concentrations (fC_{max} range: 6.25–3000 mg/L) and dosing regimens (30 different regimens) used against any organism and is the first to specifically use a dose-fractionation design to examine the PK/PD index driving bacterial activity and the emergence of resistance for fosfomycin against *P. aeruginosa*. In agreement with the *E. coli* studies of VanScoy *et al.*[41] and Lepak *et al.*,[54] bacterial killing was most closely correlated with *f*AUC/MIC. Although we have previously shown using both MDR and non-MDR isolates of *P. aeruginosa* that bacterial killing by fosfomycin against this organism is time dependent,[23] we were unable to find a relationship between activity and $fT_{>1\times MIC}$. This may be explained by a large number of the dosing regimens having a $fT_{>1\times MIC}$ of 100%. To account for this we also analysed each index at 10× MIC. Although a relationship was present between activity and $fT_{>10\times MIC}$ (Figure 4f), the *f*AUC/MIC nevertheless remained the index most closely associated with activity.

This is the first study to utilize a dose-fractionation design to investigate the relationship between PK/PD indices and bacterial killing for fosfomycin against *P. aeruginosa*. The *f*AUC/MIC was most closely correlated with bacterial killing. No fosfomycin exposures, including exposures well above those that are clinically achievable in plasma following intravenous administration, were able to suppress the emergence of resistant subpopulations. Our results suggest that for systemic infections involving *P. aeruginosa*, fosfomycin monotherapy will be ineffective.

Funding

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Transparency declarations

None to declare.

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Chapter 3

Synergistic meropenem-tobramycin combination dosage regimens against clinical hypermutable *Pseudomonas aeruginosa* at simulated epithelial lining fluid concentrations in a dynamic biofilm model

Preamble

Treatment of acute infective exacerbations of chronic respiratory infections caused by *P. aeruginosa* is a major treatment challenge in patients with CF, as many of the strains causing these infections are hypermutable and form biofilm. Carbapenems and aminoglycosides are commonly used antipseudomonals, however substantial gaps exist in our understanding of how to optimally use these antibiotics in combination to maximise the antibacterial activity and avoid or suppress the emergence of resistance which is a common phenomenon with hypermutable *P. aeruginosa* isolates. In addressing **Aim 2**, we systemically evaluated different dosage regimens of meropenem and tobramycin in monotherapies and combination against a hypermutable MDR, carbapenem-resistant *P. aeruginosa* isolate in the dynamic CDC biofilm reactor over 120 h. This is the first study examining the effect of clinically relevant concentration-time profiles, representing those in lung fluid, following FDA-approved daily doses of intravenous therapy. Two regimens for meropenem with differently shaped concentration-time profiles at the same *f*AUC/MIC representing standard (short-term infusion) and modified (continuous infusion) regimens and one regimen for tobramycin were tested in multiple replicates. In addition, different levels of meropenem lung fluid/plasma concentration ratios (30% and 60%) were also simulated to explore the impact on antibacterial effect of different levels of reported penetration into lung fluid following intravenous administration. The findings from this Chapter supported **Hypothesis 2** that against both planktonic and biofilm-embedded bacteria, continuous infusion of meropenem in combination with tobramycin provides enhanced bacterial killing and suppression of resistant mutants compared to combinations containing standard intermittent short-term infusions of meropenem. The results of this Chapter have been published in the ***Antimicrobial Agents and Chemotherapy***, as such they are presented in the format of a published article in the section immediately following.

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PHARMACOLOGY

Synergistic Meropenem-Tobramycin Combination Dosage Regimens against Clinical Hypermutable *Pseudomonas aeruginosa* at Simulated Epithelial Lining Fluid Concentrations in a Dynamic Biofilm Model

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ABSTRACT Exacerbations of chronic *Pseudomonas aeruginosa* infections are a major treatment challenge in cystic fibrosis due to biofilm formation and hypermutation. We aimed to evaluate different dosage regimens of meropenem and tobramycin as monotherapies and in combination against hypermutable carbapenem-resistant *P. aeruginosa*. A hypermutable *P. aeruginosa* isolate (meropenem and tobramycin MICs, 8 mg/liter) was investigated in the dynamic CDC biofilm reactor over 120 h. Regimens were meropenem as the standard (2 g every 8 h, 30% epithelial lining fluid [ELF] penetration) and as a continuous infusion (CI; 6 g/day, 30% and 60% ELF penetration) and tobramycin at 10 mg/kg of body weight every 24 h (50% ELF penetration). The time courses of totally susceptible and less-susceptible bacteria and MICs were determined, and antibiotic concentrations were quantified by liquid chromatography-tandem mass spectrometry. All monotherapies failed, with the substantial regrowth of planktonic (>6 $\log_{10}$ CFU/ml) and biofilm (≥6 $\log_{10}$ CFU/cm^2) bacteria occurring. Except for the meropenem CI (60% ELF penetration), all monotherapies amplified less-susceptible planktonic and biofilm bacteria by 120 h. The meropenem standard regimen with tobramycin caused initial killing followed by considerable regrowth with resistance (meropenem MIC, 64 mg/liter; tobramycin MIC, 32 mg/liter) for planktonic and biofilm bacteria. The combination containing the meropenem CI at both levels of ELF penetration synergistically suppressed the regrowth of total planktonic bacteria and the resistance of planktonic and biofilm bacteria. The combination with the meropenem CI at 60% ELF penetration, in addition, synergistically suppressed the regrowth of total biofilm bacteria. Standard regimens of meropenem and tobramycin were ineffective against planktonic and biofilm bacteria. The combination with meropenem CI exhibited enhanced bacterial killing and resistance suppression of carbapenem-resistant hypermutable *P. aeruginosa*.

KEYWORDS combination therapy, hypermutators, biofilm infections, antibiotic resistance, dosage regimens

Carbapenem-resistant *Pseudomonas aeruginosa* has been classified by the World Health Organization as one of the top three critical pathogens requiring new antibiotic treatments (1). *P. aeruginosa* has a particularly large armamentarium of resistance mechanisms and can develop resistance against virtually all antibiotics in

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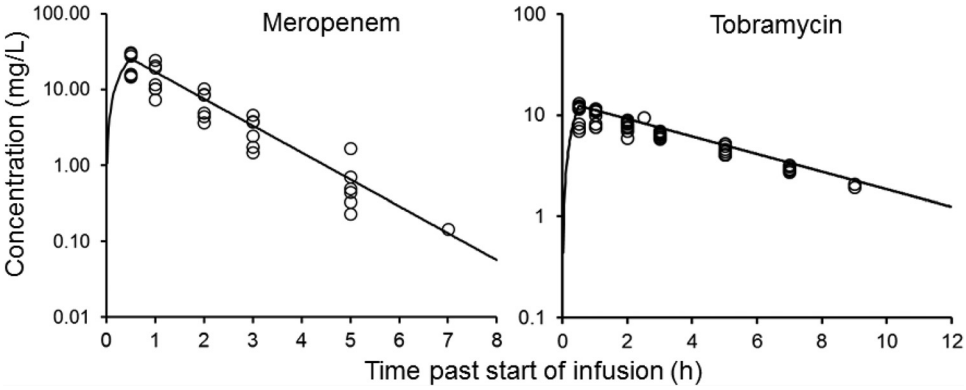

FIG 1 Pharmacokinetic profiles showing the relationship between the targeted (lines) and measured (symbols) meropenem and tobramycin concentrations in the CBR.

monotherapy. Such resistance development often results in treatment failure (2, 3). Respiratory infections caused by *P. aeruginosa* are a serious clinical problem for patients with cystic fibrosis (CF). Acute infective exacerbations (AIE) of chronic *P. aeruginosa* infections cause progressive lung function decline followed by respiratory failure (4, 5). Thus, they are a main driver of early death for patients with CF. Indeed, the rates of multidrug-resistance (MDR) for these infections in CF patients are substantially higher than those in patients in an intensive care unit (6).

The ability of *P. aeruginosa* to become hypermutable and to form a biofilm renders AIE especially difficult to treat (7, 8). Hypermutable isolates (i.e., those with an up to ~1,000-fold increased mutation rate due to defects in DNA repair or error avoidance systems) account for up to ~54% of *P. aeruginosa* strains in CF respiratory infections and are associated with reduced lung function (8–10). Despite an increased mutation rate, hypermutable strains generally do not show reduced fitness in the nutrient-rich environment of the CF lung. Their carriage is highly correlated with MDR, and hypermutation is important for biofilm development (8, 11). The formation of a biofilm hampers antibiotic effectiveness, e.g., via extracellular matrix formation, which reduces antibiotic penetration. Biofilm growth also leads to greater phenotypic diversity and, thus, a greater persistence of infections (12, 13). Therefore, biofilm-associated infections by hypermutable *P. aeruginosa* are extremely difficult to treat, especially when the hypermutable strains are MDR.

Current antibiotic regimens against *P. aeruginosa* infections in patients with CF are suboptimal; monotherapy is often ineffective, and combination regimens are used empirically (4, 14). We have demonstrated synergistic bacterial killing and the suppression of resistance emergence in hypermutable *P. aeruginosa* with a modified combination regimen of meropenem and tobramycin in the dynamic hollow-fiber infection model (HFIM) (15). However, treatment regimens against hypermutable *P. aeruginosa* have never been evaluated in a dynamic biofilm model, such as the Centers for Disease Control and Prevention biofilm reactor (CBR), that allows examination of antibacterial effects against both planktonic and biofilm bacteria. Thus, the aim of the present study was to use the CBR to simulate the concentration-time profiles for different meropenem and tobramycin dosage regimens observed in the epithelial lining fluid (ELF) of patients with CF when given as monotherapy and in combination and characterize the bacterial killing and resistance suppression of carbapenem-resistant hypermutable *P. aeruginosa*.

RESULTS

Pharmacokinetic validation, bacterial killing, and emergence of resistance. The pharmacokinetic profiles observed in the CBR (Fig. 1) were in good agreement with the targeted exposures (Table 1). The observed meropenem and tobramycin concentrations were, on average, within 10% of the targeted concentrations. The viable count profiles for planktonic and biofilm bacteria are presented in Fig. 2. The counts on

TABLE 1 Clinically representative ELF concentrations, exposures, and pharmacokinetic/pharmacodynamic indices for meropenem and/or tobramycin in the CBR[a]

Treatment	fC_{max}/fC_{min} or fC_{ss} (mg/liter)	$fAUC_{24}$ (mg·h/liter)	fC_{max}/MIC	$fT_{>MIC}$ (%)	$fAUC_{24}$/MIC
MER at 2 g every 8 h	25.4/0.06	115	3.18	22	
MER at 6 g/day as a CI	4.79	115	0	0	
MER at 6 g/day as a CI (60% ELF penetration)	9.58	230	1.20	100	
TOB at 10 mg/kg every 24 h	12.3/0.11	64.4	1.54		8.05

[a]MER, meropenem; TOB, tobramycin; CI, continuous infusion; fC_{max}, unbound maximum concentration; fC_{min}, unbound minimum concentration before the next dose; fC_{ss}, unbound average steady-state concentration; $fAUC_{24}$, the area under the unbound concentration-time curve over 24 h; fC_{max}/MIC, the ratio of the fC_{max} to the MIC; $fT_{>MIC}$, the cumulative percentage of a 24-h period that the unbound concentrations exceeded 1× MIC; $fAUC_{24}$/MIC, the ratio of the $fAUC_{24}$ to the MIC. The simulated half-lives were 0.8 h for meropenem and 3.5 h for tobramycin. No loading dose was administered for intermittent dosing, whereas the modified meropenem dosage regimen (6-g/day CI) was started at the fC_{ss} of either 4.79 mg/liter (30% ELF penetration) or 9.58 mg/liter (60% ELF penetration). The simulated ELF penetration was 30% for meropenem, unless it is specified to be 60%, and was 50% for tobramycin.

antibiotic-containing agar are shown in Fig. 3, log changes in viable counts are shown in Table 2, mutation frequencies are shown in Table 3, and baseline and endpoint MICs are shown in Table 4.

Planktonic bacteria. The starting inoculum (mean ± standard error [SE]) in all arms was 7.19 ± 0.05 $\log_{10}$ CFU/ml ($n = 14$). *P. aeruginosa* CW8 in the control chambers grew to 7.75 ± 0.15 $\log_{10}$ CFU/ml by 24 h, and the growth plateaued at ~8.1 $\log_{10}$ CFU/ml (Fig. 2A). Colonies on drug-containing agar increased approximately in proportion to the growth of the total bacterial population (Fig. 3A, C, E, and G and Table 3).

Meropenem monotherapy simulating the standard regimen (2 g every 8 h) resulted in ~3-$\log_{10}$-CFU/ml initial killing at 3 h, followed by steady regrowth to ~7 $\log_{10}$ CFU/ml at 48 h, with slower regrowth toward control values occurring thereafter (Fig. 2A and Table 2). With this regimen, less-susceptible populations increased rapidly, with approximately half of the entire population growing on agar containing meropenem at 6 mg/liter by 120 h. Similar increases were observed on agar containing meropenem at 15 mg/liter, although growth remained ~1 log lower than the total population at 120 h. Emergence of resistance was observed, with an ~2.5-log increase of the meropenem-resistant bacteria compared to that for the growth control being seen at 120 h (Fig. 3A and C and Table 3). The MIC of colonies recovered from 15-mg/liter meropenem-containing plates at this time point was 128 mg/liter (Table 4). The bacterial killing achieved with the modified meropenem regimen at 6 g/day as a continuous infusion (CI) simulating 30% and 60% ELF penetration closely matched that achieved with the standard regimen (2 g every 8 h) over the first 24 h, but thereafter growth remained ~1 $\log_{10}$ CFU/ml (30% ELF penetration) and ~2 $\log_{10}$ CFU/ml (60% ELF penetration) lower than that for the standard regimen; for the treatment simulating 60% ELF penetration, regrowth at 120 h reached ~6 $\log_{10}$ CFU/ml (Fig. 2A and Table 2). While the increases in less-susceptible populations with the CI simulating 30% ELF penetration closely matched those for the standard regimen, the less-susceptible populations remained suppressed for 60% ELF penetration (Fig. 3A and C and Table 3). By 72 h with the latter treatment, growth on agar containing meropenem at 6 mg/liter and 15 mg/liter was ~2 and <1 $\log_{10}$ CFU/ml, respectively. Furthermore, the emergence of resistance was not observed at 120 h, and the MICs of colonies recovered from drug plates were increased by only 1 dilution (MIC, 16 mg/liter) (Table 4). Amplification of resistance with meropenem 30% ELF penetration was more evident, with an ~2.1-log increase in the meropenem-resistant population at 120 h in comparison to that for the growth control (Fig. 3C and Table 3). The MIC at this time point was 64 mg/liter (Table 4). The tobramycin monotherapy produced rapid (within the first 7 h) initial killing of ~3 $\log_{10}$ CFU/ml, followed by steady regrowth, such that growth approximated that of the growth control by 72 h (Fig. 2C and Table 2). Amplification of bacteria less susceptible to tobramycin was observed. The proportion of colonies growing on tobramycin-containing agar increased substantially over 120 h, with a large proportion of the entire population growing on plates containing 3 mg/liter tobramycin; on plates containing 7.5 mg/liter tobramycin, growth increased to within ~2

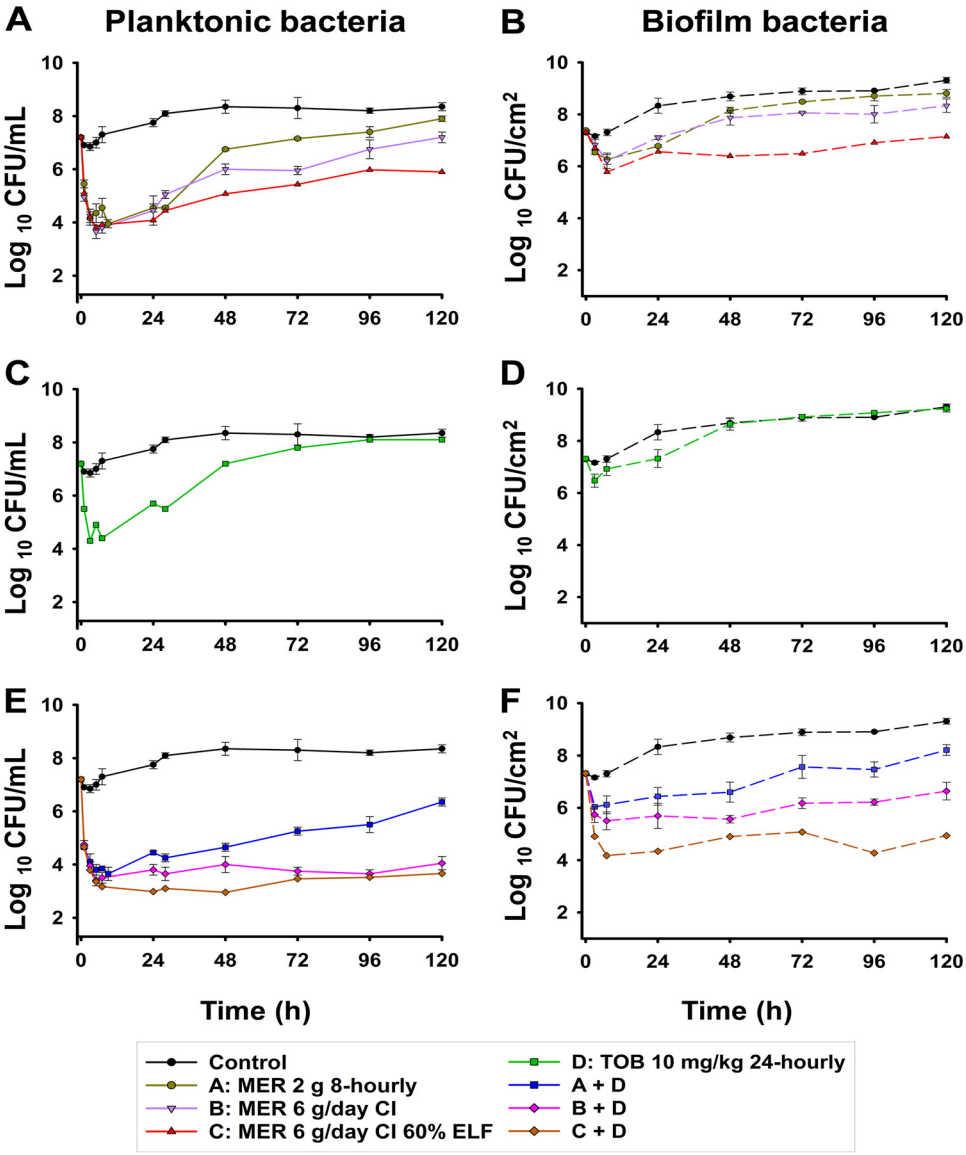

FIG 2 Total viable counts for the growth control and treatments with meropenem (MER) and/or tobramycin (TOB) at clinically relevant ELF concentration-time profiles sampled from the medium within the reactor, i.e., planktonic bacteria receiving meropenem monotherapy (A), tobramycin monotherapy (C), or the combination of meropenem and tobramycin (E), and from coupons, i.e., biofilm bacteria receiving meropenem monotherapy (B), tobramycin monotherapy (D), or the combination of meropenem and tobramycin (F). The results are presented as the average ± SE. The *y* axis starts from the limit of counting.

$\log_{10}$ CFU/ml of the total population (Fig. 3E and G and Table 3). The MIC at 120 h was 64 mg/liter (Table 4).

The combination containing the standard meropenem regimen produced ~3.3-$\log_{10}$-CFU/ml initial killing at 5 h and regrowth to within ~2 $\log_{10}$ CFU/ml of the growth of the growth control at 120 h (Fig. 2E and Table 2). The amplification of less-susceptible and resistant bacteria in comparison to that of the growth control was observed. Less-susceptible populations increased dramatically, such that virtually the entire population at 120 h grew on agar containing meropenem at 6 mg/liter and tobramycin at 3 mg/liter (Fig. 3A and E and Table 3). The MIC at this time point was 64 mg/liter for meropenem and 32 mg/liter for tobramycin (Table 4).

The combination treatment with the modified meropenem regimen with 30% ELF penetration produced initial killing of ~3.8 $\log_{10}$ CFU/ml at 5 h, while that with 60% ELF penetration produced initial killing of ~4.4 $\log_{10}$ CFU/ml at 24 h. Synergistic bacterial

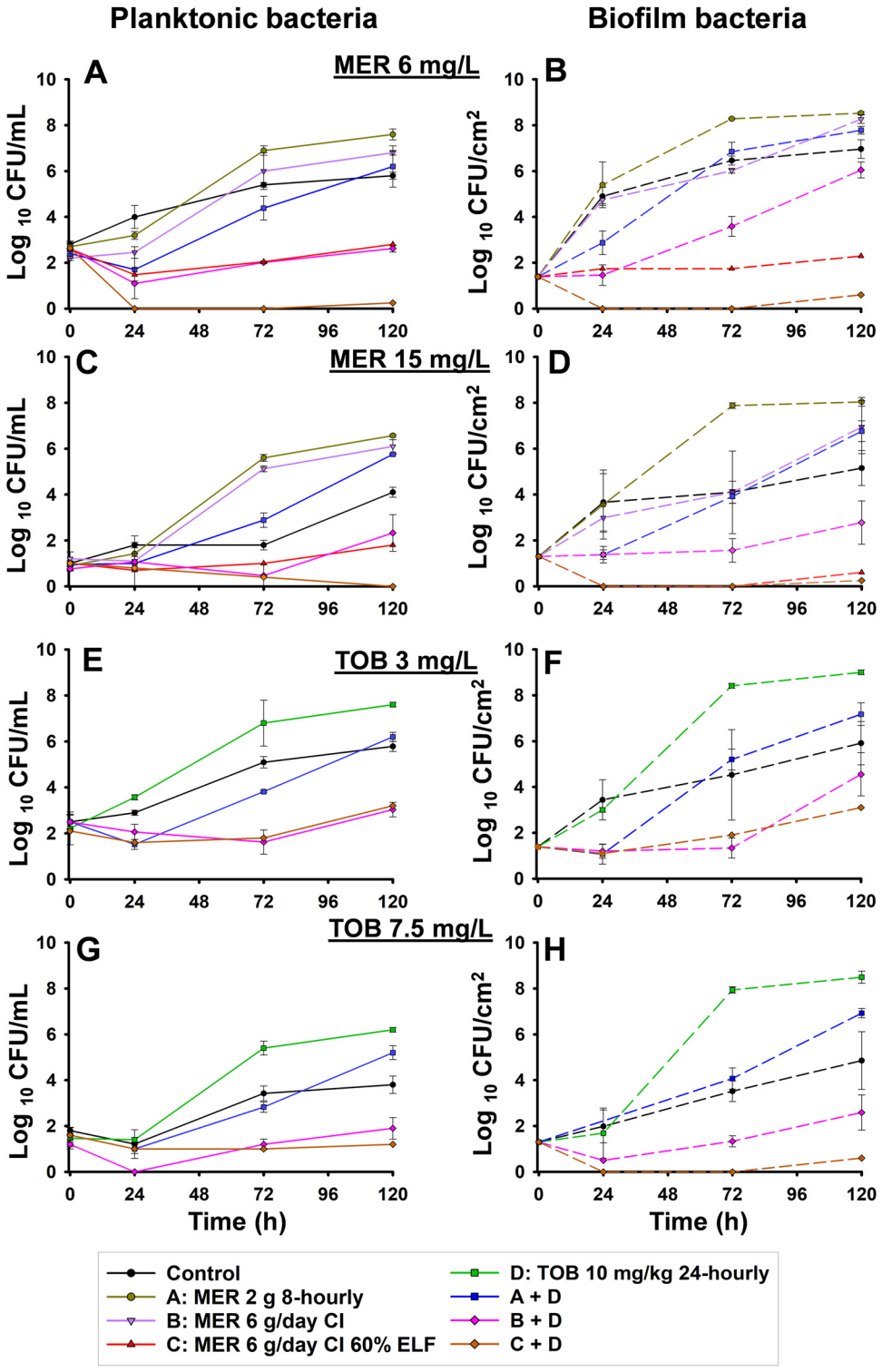

FIG 3 Effect of each dosage regimen on the counts of bacteria able to grow on agar plates containing 6 or 15 mg/liter of meropenem or 3 or 7.5 mg/liter of tobramycin. The results are represented as the average ± SE. To differentiate less-susceptible subpopulations from the predominant population, the antibiotic concentrations in agar were based upon Etest MICs, which were 1.5 mg/liter for meropenem and 0.75 mg/liter for tobramycin (9).

killing (≥2 $\log_{10}$ CFU/ml) was observed from 28 or 72 h onwards with both levels of ELF penetration; regrowth remained suppressed at ~3.5 to 4 $\log_{10}$ CFU/ml at 120 h (Fig. 2E and Table 2). However, differences in the regrowth of less-susceptible populations were observed. On agar containing meropenem at 15 mg/liter, regrowth of ~2 $\log_{10}$ CFU/ml

TABLE 2 Log changes in viable cell counts at various time points with clinically relevant ELF concentrations of meropenem and/or tobramycin[a]

Time (h)	Log change= [$\log_{10}(CFU_t) - \log_{10}(CFU_0)$]													
	A: MER 2g Q8h		B: MER 6g/d CI		C: MER 6g/d CI 60% ELF		D: TOB 10mg/kg Q24h		A + D		B + D		C + D	
	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria
1	-1.95		-2.25		-1.82		-1.70		-2.45		-2.45		-2.74	
3	-3.25	-0.85	-2.95	-0.47	-2.70	-0.62	-2.85	-0.43	-3.00	-1.28	-3.25	-1.34	-3.61	-2.41
5	-3.05		-3.55		-3.09		-2.70		-3.30		-3.80		-4.04	
7	-2.85	-1.02	-3.15	-1.58	-2.99	-0.74	-2.90	0.02	-3.25	-1.20	-3.70	-1.58	-4.23	-3.15
24	-2.85	-0.61	-2.75	-0.19	-2.82	-0.56	-1.80	0.41	-2.65	-0.88	-3.40	-1.69	-4.42	-2.98
28	-2.85		-2.15		-2.45		-1.35		-2.85		-3.55		-4.30	
48	-0.65	0.77	-1.20	0.58	-1.52	-0.92	-0.15	1.75	-2.45	-0.72	-3.20	-1.55	-4.45	-2.42
72	-0.25	1.10	-1.25	0.76	-1.47	-0.83	0.60	2.02	-1.85	0.25	-3.48	-0.90	-3.94	-2.25
96	0.10	1.32	-0.45	0.71	-0.92	-0.41	0.80	2.17	-1.60	0.15	-3.55	-0.86	-3.88	-3.05
120	0.60	1.42	-0.10	1.04	-1.00	-0.17	0.90	2.34	-0.75	0.89	-3.15	-0.44	-3.74	-2.38

[a]MER, meropenem; TOB, tobramycin; CFU_t, number of CFU at time *t*; CFU_0, number of CFU at time zero. The green background indicates synergy (a $\geq$2-$\log_{10}$ decrease in the number of CFU per milliliter or the number of CFU per square centimeter with the combination compared to the value for its most active component); the blue background indicates a 1.0- to <2-$\log_{10}$ decrease in the number of CFU per milliliter or the number of CFU per square centimeter with the combination compared to the value for its most active component.

was observed at 120 h with the combination simulating 30% ELF penetration of the meropenem CI (MIC, 32 mg/liter). However, virtually no colonies were detected from 24 h onwards at either meropenem plate concentration with the combination simulating 60% ELF penetration of meropenem. Growth on agar containing tobramycin at 7.5 mg/liter was ~1.5 to 2 $\log_{10}$ CFU/ml at 120 h for both combination treatments involving the meropenem CI, which was ~2 $\log_{10}$ CFU/ml below the growth control counts (Fig. 3G).

Biofilm-embedded bacteria. In the growth control, biofilm bacteria grew steadily to ~9 $\log_{10}$ CFU/cm^2 by 72 h and plateaued until 120 h (Fig. 2B); moderate increases

TABLE 3 Log_{10} mutation frequencies at 6 mg/liter and 15 mg/liter meropenem and 3 mg/liter and 7.5 mg/liter tobramycin for each simulated regimen

Time (h)	Arm	MEROPENEM				TOBRAMYCIN			
		6 mg/L		15 mg/L		3 mg/L		7.5 mg/L	
		Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria
0	All Regimens	-4.74	-5.93	-6.00	-6.02	-4.50	-5.93	-5.70	-5.82
24	Control	-3.87	-3.38	-5.77	-5.63	-4.97	-4.85	-5.60	-5.67
	A: MER 2g Q8h	-1.36	-1.38	-3.13	-3.21				
	B: MER 6g/d CI	-2.60	-2.36	-3.95	-4.12				
	C: MER 6g/d CI 60% ELF	-2.97	-4.16	-3.75	-6.76				
	D: TOB 10mg/kg Q24h					-1.98	-3.67	-4.15	-4.98
	A + D	-2.60	-3.57	-3.30	-5.06	-2.78	-4.37	-3.30	-5.44
	B + D	-2.24	-3.91	-2.27	-3.99	-1.27	-4.16	-3.33	-5.37
	C + D	-3.21	-4.34	-3.21	-4.34	-1.61	-3.24	-2.21	-4.34
72	Control	-2.80	-2.34	-5.40	-4.70	-3.11	-4.27	-4.78	-5.28
	A: MER 2g Q8h	-0.51	-0.20	-1.80	-0.89				
	B: MER 6g/d CI	-0.75	-2.04	-1.62	-3.96				
	C: MER 6g/d CI 60% ELF	-3.84	-4.75	-4.88	-6.49				
	D: TOB 10mg/kg Q24h					-1.30	-0.51	-2.70	-0.98
	A + D	-1.12	-0.72	-2.62	-3.55	-1.69	-2.36	-2.68	-3.49
	B + D	-1.39	-3.04	-2.93	-4.73	-1.78	-5.29	-2.20	-5.30
	C + D	-3.52	-5.07	-3.52	-5.07	-1.72	-3.17	-2.52	-5.07
120	Control	-2.57	-2.23	-4.27	-4.04	-2.58	-3.27	-4.57	-4.33
	A: MER 2g Q8h	-0.35	-0.28	-1.33	-0.61				
	B: MER 6g/d CI	-0.40	-0.07	-1.00	-1.39				
	C: MER 6g/d CI 60% ELF	-3.30	-4.86	-4.10	-6.55				
	D: TOB 10mg/kg Q24h					-0.45	-0.24	-1.85	-0.75
	A + D	-0.15	-0.38	-0.59	-1.34	-0.15	-1.03	-1.15	-1.28
	B + D	-1.11	-0.66	-1.40	-3.93	-0.70	-2.15	-2.58	-4.11
	C + D	-3.41	-4.33	-3.66	-4.68	-0.46	-1.83	-2.46	-4.33

[a]MER, meropenem; TOB, tobramycin. The red background indicates a high mutation frequency, i.e., a large proportion of less-susceptible bacteria being present in the total population; the green background indicates a low mutation frequency, i.e., a small proportion of less-susceptible bacteria being present in the total population.

TABLE 4 MIC values for colonies obtained from antibiotic-containing agar plates at 0 and 120 h for each dosage regimen[a]

Treatment	Meropenem at 15 mg/liter			Tobramycin at 7.5 mg/liter		
		MIC (mg/liter)			MIC (mg/liter)	
	Time (h)	Planktonic bacteria	Biofilm bacteria	Time (h)	Planktonic bacteria	Biofilm bacteria
Control	0	16	8	0	16	8
	120	32	16	120	32	16
MER at 2 g every 8 h	120	128	32	—	—	—
MER at 6 g/day as a CI	120	64	32	—	—	—
MER at 6 g/day as a CI (60% ELF penetration)	120	16	NC	—	—	—
TOB at 10 mg/kg every 24 h	—	—	—	120	64	32
MER at 2 g every 8 h + TOB at 10 mg/kg every 24 h	120	64	32	120	32	32
MER at 6 g/day as a CI + TOB at 10 mg/kg every 24 h	120	32	16	120	32	16
MER at 6 g/day as a CI (60% ELF penetration) + TOB at 10 mg/kg every 24 h	120	NC	NC	120	NC	NC

[a]The agar plates contained meropenem (MER) at 15 mg/liter and tobramycin (TOB) at 7.5 mg/liter. NC, no colonies grew on the antibiotic-containing plates; —, not tested.

in the proportion of less-susceptible populations were observed across 120 h (Fig. 3B, D, F, and H and Table 3). Following 7 h of treatment, bacterial killing from all monotherapy regimens was ~1 $\log_{10}$ CFU/cm^2. After that, regrowth occurred with all regimens except the meropenem CI with 60% ELF penetration, such that growth was within ≤2 $\log_{10}$ CFU/cm^2 of that of the control from 48 h onwards; with the CI (60% ELF penetration), growth remained at ~6.5 to 7.0 $\log_{10}$ CFU/cm^2 across 120 h (Fig. 2B and D and Table 2). Substantial increases in less-susceptible populations occurred with the tobramycin and both the standard and CI (30% ELF penetration) meropenem regimens but not with the CI (60% ELF penetration) meropenem regimen (Fig. 3B, D, F, and H and Table 3). With the latter regimen, growth on agar containing meropenem at 6 mg/liter remained steady at ~2 $\log_{10}$ CFU/cm^2 across 120 h, whereas virtually no colonies were detected on agar containing meropenem at 15 mg/liter from 24 h onwards. At 120 h, the MIC was 32 mg/liter for the meropenem standard regimen and the CI at 30% ELF penetration; the MIC for the tobramycin regimen was also 32 mg/liter (Table 4).

The combination containing the standard meropenem regimen simulating 30% ELF penetration produced ~1-$\log_{10}$-CFU/cm^2 initial killing at 3 h, followed by regrowth to within ~1 $\log_{10}$ CFU/cm^2 of the growth of the growth control at 120 h (Fig. 2F and Table 2). Amplification of bacteria less susceptible to meropenem and tobramycin in comparison to that of the growth control was observed (Fig. 3B, D, F, and H and Table 3). The MIC for both meropenem and tobramycin was 32 mg/liter at 120 h (Table 4). In contrast, with the combination regimens simulating the meropenem CI at 30% or 60% ELF penetration, a more substantial antibacterial effect was observed. With the meropenem CI at 30% ELF penetration, biofilm bacteria remained suppressed below ~6 $\log_{10}$ CFU/cm^2 up to 48 h, with only ~1 $\log_{10}$ CFU/cm^2 of growth occurring thereafter (Fig. 2F and Table 2). At 24 and 48 h, bacterial counts were 1 to 2 $\log_{10}$ CFU/cm^2 lower for the combination than for the most active monotherapy. Bacteria less susceptible to meropenem and tobramycin grew to within ~2 $\log_{10}$ CFU/cm^2 of the growth of the growth control by 120 h (Fig. 3B, D, F, and H). The MIC for meropenem and tobramycin was 16 mg/liter at this time point (Table 4). The combination simulating the meropenem CI at 60% ELF penetration achieved ~2.3- to 3.2-$\log_{10}$-CFU/cm^2 bacterial killing from 7 h onwards (Fig. 2F and Table 2); synergistic bacterial killing (≥2 $\log_{10}$ CFU/cm^2 compared to the killing obtained with the most active monotherapy) was observed at 24, 96, and 120 h (Fig. 2B and F and Table 2). A negligible number (~0.5 $\log_{10}$ CFU/cm^2) of colonies was observed on plates containing tobramycin at 7.5 mg/liter or meropenem at 6 or 15 mg/liter (Fig. 3B, D, F, and H).

DISCUSSION

This study systematically investigated the bacterial killing and resistance suppression of standard versus modified dosage regimens of meropenem and tobramycin

against a carbapenem-resistant clinical hypermutable *P. aeruginosa* isolate in the CBR. The pharmacokinetic profiles simulated were representative of the unbound antibiotic concentrations expected in the ELF of patients with CF.

Pharmacokinetic/pharmacodynamic approaches to optimize the administration of β-lactams, including meropenem, traditionally involve maximizing the cumulative percentage of a 24-h period that the unbound concentrations exceed 1× MIC ($fT_{>MIC}$) for the infecting pathogen (16, 17). For serious bacterial infections, targets such as 100% $fT_{>4-5\times MIC}$ (18–20) have been proposed. The $fT_{>MIC}$ can be modulated by altering the mode of administration. In the present investigations, meropenem was delivered either as a short-term infusion (standard regimen) or as a continuous infusion (CI; modified regimen), representing the extreme modes of administration in clinical practice. Importantly, however, the above-mentioned targets relate to planktonic bacteria; targets for biofilm bacteria are yet to be established and likely to be higher.

In our CBR studies, all meropenem regimens in monotherapy, at both levels of ELF penetration, were unable to suppress regrowth to below ~6 $\log_{10}$ for both planktonic and biofilm bacteria (Fig. 2). Even the CI with 60% ELF penetration was not successful, despite achieving a 100% $fT_{>MIC}$. This comprehensive failure of meropenem regimens strongly argues against the use of meropenem as monotherapy against a meropenem-resistant hypermutable *P. aeruginosa* isolate. For a meropenem-susceptible hypermutable *P. aeruginosa* strain, we have previously demonstrated in a 10-day HFIM study that the CI of meropenem to achieve a concentration as high as ~8× MIC was unable to suppress the emergence of less-susceptible planktonic bacteria (15). In the current study, biofilm bacteria were more resilient to meropenem than planktonic bacteria (Fig. 2). The bacterial cells in a biofilm are difficult to kill because of multiple factors. This includes the low metabolic activity of subpopulations located in the inner parts of the biofilm; e.g., low peptidoglycan production affects bacterial killing by meropenem (21). In a recent study of *P. aeruginosa* in a biofilm, meropenem concentrations substantially higher than those expected in ELF achieved some activity against meropenem-susceptible strains, while there was no activity against a meropenem-resistant strain (22).

For aminoglycosides, such as tobramycin, the ratio of exposure across a 24-h period to the MIC (the area under the unbound concentration-time curve [*f*AUC]/MIC) and the ratio of the unbound maximum concentration (fC_{max}) to the MIC (fC_{max}/MIC) have been correlated with antibacterial activity. An *f*AUC/MIC of >70 and an fC_{max}/MIC of 8 to 10 have been proposed as clinical targets (23); in the present study, the corresponding values were ~8 and ~1.5 (Table 1). For cationic antimicrobials, such as tobramycin, the presence of extracellular DNA in the biofilm decreases activity via chelation (24, 25). Thus, it is not surprising that the tobramycin regimen was ineffective in suppressing regrowth, resulting in a large increase of less-susceptible planktonic and biofilm bacteria (Fig. 2 and 3). This result was in agreement with that of our previous *in vitro* study, where tobramycin monotherapy failed to inhibit the regrowth of planktonic hypermutable *P. aeruginosa* even for *f*AUC/MIC values of 72 and 168 (26).

In the CBR, the combination containing the standard meropenem regimen (30% ELF penetration) with tobramycin resulted in a significant regrowth of less-susceptible planktonic and biofilm bacteria. Total bacterial counts were within 1 log of those achieved with the most active monotherapy at each time point. Importantly, the combination containing the modified meropenem (CI) regimen achieved enhanced bacterial killing and resistance suppression. This combination suppressed the regrowth and resistance of planktonic bacteria over 5 days at both levels of ELF penetration, demonstrating clear synergy. Although some regrowth of total biofilm bacteria was observed from 48 h onwards when simulating 30% ELF penetration of meropenem, resistant subpopulations remained suppressed compared to the growth control over 5 days. It is not surprising that the combination with standard meropenem dosing was less effective than that with the meropenem CI, as in the former case there were substantial periods with essentially no antibiotic present for activity. The combination simulating 60% ELF penetration synergistically suppressed the regrowth of both total

and resistant biofilm bacteria over 5 days. The synergy observed was notable, given that the isolate was meropenem resistant and tobramycin intermediate.

The synergistic bacterial killing and suppression of resistance observed in our study may be due to the different mechanisms of action and resistance of each antibiotic. Meropenem inhibits cell wall synthesis via binding to penicillin-binding proteins (27), and the main mechanisms of resistance in *P. aeruginosa* involve AmpC β-lactamase overexpression, reduced outer membrane porin OprD, and enzymatic inactivation via carbapenemases (28). Tobramycin predominantly acts by protein synthesis inhibition (29) but also by the disruption of the outer bacterial membrane (30–32). Resistance mechanisms against tobramycin include target site modification, enzymatic cleavage, increased expression of MexXY-OprM, and reduced outer membrane permeability (33, 34). For CW8, we previously identified mutations in genes related to both meropenem (*oprD*, *ampC*, *ampR*) and tobramycin (*fusA1*) resistance (9). Although the effects of each of the antibiotics in the combination were attenuated due to resistance, our results indicate that exposing the bacteria to both antibiotics simultaneously had a beneficial effect. In addition, mechanistic synergy may have been caused by tobramycin enhancing the target site penetration of meropenem (15). We have demonstrated that tobramycin in combination with another carbapenem, imipenem, caused extensive ultrastructural disruption of the outer membrane (32). This mechanistic synergy may apply not only to planktonic bacteria but also to biofilm bacteria.

To the best of our knowledge, this is the first study to examine the activity of the meropenem and tobramycin combination against carbapenem-resistant hypermutable *P. aeruginosa* in planktonic and biofilm growth by simulating ELF pharmacokinetics. Two studies in the dynamic HFIM (15, 18) and one *in vivo* infection model study (35) also examined the activity of this combination against *P. aeruginosa*. It is important to note that these studies involved susceptible isolates and investigated only planktonic growth, and the *in vitro* studies represented plasma rather than ELF concentrations. Two static concentration time-kill studies previously examined the activity of the same combination at a range of concentrations against susceptible planktonic *P. aeruginosa* (36, 37). The effect of the meropenem-tobramycin combination on the biofilm biomass of a susceptible *P. aeruginosa* strain has also been studied using a dynamic flow cell model and microscopy (38). However, that study did not quantify the counts of either biofilm or planktonic bacteria, nor did it examine the emergence of resistance, and the concentrations were higher than those achievable in ELF after intravenous dosing.

The current study has a number of strengths. It is the first study to examine the activity of the meropenem-tobramycin combination against a carbapenem-resistant hypermutable *P. aeruginosa* isolate. Furthermore, this is the only dynamic *in vitro* study to utilize concentration-time profiles representative of those in ELF for this combination. Since different levels of ELF penetration of meropenem have been reported (39–41), we examined both 30% and 60% ELF penetration. Importantly, the combination with the modified meropenem regimen achieved enhanced bacterial killing and resistance suppression even at the low ELF penetration. This study was conducted over 5 days of treatment, quantified both biofilm and planktonic bacteria, and evaluated the emergence of resistance. In addition, multiple biological replicates were performed to demonstrate the reproducibility of the total and less-susceptible bacterial counts. It is also important to note some limitations. The comprehensive studies conducted involved one clinical *P. aeruginosa* isolate. Future studies may be directed at evaluating the effect of the combination against other isolates, investigating its effect on the biofilm structure via confocal microscopy, and developing a mechanism-based mathematical model for antibiotic effects on biofilm bacteria. In addition, as with all other *in vitro* models, the CBR lacks an immune system. Therefore, future animal studies may be warranted to assess immune system effects on residual populations following the initial bacterial killing by the antibiotics. However, the accurate representation of humanized pharmacokinetic profiles is challenging in animal models due to the differences in clearance and half-life. In addition, given the ethical limitations on study duration inherent in animal studies, suppression of the emergence of resistance, a key compo-

nent of the present study, is best examined in the CBR, where longer study durations can be employed.

In conclusion, standard regimens of meropenem and tobramycin, both as monotherapy and in combination, were ineffective in suppressing regrowth and the emergence of resistance in both planktonic and biofilm bacteria. Importantly, however, the combination with the meropenem continuous infusion regimen, at both levels of ELF penetration, exhibited enhanced bacterial killing and resistance suppression against carbapenem-resistant hypermutable *P. aeruginosa*. Thus, this promising combination regimen warrants further evaluation.

MATERIALS AND METHODS

Bacterial isolate, antibiotics, and MICs. A previously described clinical hypermutable *P. aeruginosa* isolate (CW8) was employed (9). Hypermutability was defined as a mutation frequency on rifampin-containing agar at least 20-fold higher than that obtained for the control strain, PAO1 (9, 10). Sterile stock solutions of meropenem (lot Maus1025; Kabi, Melbourne, Australia) and tobramycin (lot LC24138; AK Scientific, Union City, MD, USA) were prepared in Milli-Q water immediately prior to each experiment. The MICs determined in duplicate on separate days using agar dilution per Clinical and Laboratory Standards Institute (CLSI) guidelines were 8 mg/liter for each antibiotic (42). Susceptibility and resistance were defined as MICs of ≤2 mg/liter and ≥8 mg/liter, respectively, for meropenem, and ≤4 mg/liter and ≥16 mg/liter, respectively, for tobramycin, per CLSI guidelines (42). The isolate was resistant to meropenem, intermediate to tobramycin, and MDR, based on agar dilution MICs. MDR was defined as nonsusceptibility to at least one antimicrobial agent in three or more antimicrobial categories (43). The biofilm formation capacity of CW8 was confirmed by the crystal violet assay.

***In vitro* dynamic biofilm model, quantification of bacterial killing, emergence of resistance, and dosage regimens.** The time courses of bacterial killing and the emergence of resistance of planktonic and biofilm-embedded bacteria for the standard and modified regimens of meropenem and tobramycin as monotherapy and in combination were investigated over 120 h using the CBR (Bio Surface Technologies, Bozeman, MT, USA). The CBR model consisted of a 1-liter glass reactor connected to a 10-liter carboy containing sterile drug-free cation-adjusted Mueller-Hinton broth (CAMHB; BD, Sparks, MD, USA) containing 25 mg/liter Ca^{2+}, 12.5 mg/liter Mg^{2+}, and 1% tryptic soy broth (TSB) (CAMHB–1% TSB). Broth was pumped through the model (broth volume in the reactor, 350 ml), along with mixing, and shear was generated by a magnetic stir bar operating at 130 rpm. A hot plate maintained the CAMHB–1% TSB at 35°C. The biofilm formed on removable polycarbonate coupons (diameter, 12.7 mm) located in eight polypropylene coupon holders suspended from the reactor lid (three coupons per holder); the total surface area of each coupon was 2.53 cm^2.

The protocol for biofilm growth was similar to that described in our previous study (44). Prior to each experiment, CW8 was subcultured onto cation-adjusted Mueller-Hinton agar (CAMHA) containing 25 mg/liter Ca^{2+} and 12.5 mg/liter Mg^{2+} (Media Preparation Unit, University of Melbourne, Melbourne, Australia) and incubated at 35°C for 48 h. Following incubation, 2 or 3 random colonies were selected and grown overnight in 10 ml TSB, from which early-log-phase growth was obtained. A 28-h conditioning phase was then commenced via inoculation of 1 ml of this suspension into the model. Conditioning initially involved 24 h of incubation in drug-free CAMHB–1% TSB. Subsequently, all CAMHB–1% TSB was removed (to expel all planktonic bacteria and allow the amplification of bacteria shedding from the biofilm) and the reactor was refilled with drug-free CAMHB–1% TSB, pumped through the model for 4 h (flow rate, 11.67 ml/min) prior to the commencement of antibiotic treatment (i.e., 0 h) (44). The presence of biofilm on the coupons was confirmed by electron microscopy at 0 h.

At 0 h, the flow rate was changed to 4.9 ml/min for all treatments, to simulate a meropenem ELF elimination half-life ($t_{1/2}$) of ~0.8 h, reflecting that in patients with CF (45). For tobramycin-containing treatments, tobramycin was supplemented to achieve the required ELF $t_{1/2}$ of ~3.5 h (46, 47). For intermittent infusions, the antibiotics were administered using syringe drivers. The meropenem CI was achieved by administering a loading dose at 0 h directly into the reactor to immediately attain the required steady-state ELF concentration and spiking the meropenem stock solution into the carboy to maintain the steady-state concentration. Meropenem and tobramycin have negligible plasma protein binding; therefore, the concentrations used represent unbound (free) concentrations (Table 1). For meropenem, two regimens utilizing the highest FDA-recommended daily dose (6 g/day) were selected: the standard regimen of 2 g three times daily via a 30-min intravenous infusion with an fC_{max} of 25.3 mg/liter (Table 1) and a modified regimen of 6 g/day as a continuous infusion (CI) with a loading dose to rapidly achieve the unbound average steady-state concentration(fC_{ss}; Table 1). The modified regimen was simulated for both 30% and 60% ELF penetration. The pharmacokinetic profiles simulated in the CBR were based on the antibiotic concentrations over time that would be expected in the ELF of CF patients given the respective regimens. These expected unbound antibiotic concentration-time profiles were simulated *in silico* using the Berkeley Madonna (version 8.3.18) program (48), based on clinical studies and population pharmacokinetic models for CF patients (45, 46). The rate and extent of penetration of meropenem (30%) and tobramycin (50%) into ELF were derived from multiple published studies in patients (39, 41, 47). For meropenem, a higher ELF penetration (60%) based on a healthy volunteer study (40) was also considered. For tobramycin, the highest FDA-recommended daily dose for CF patients (10 mg/kg of body weight) was administered as a 30-min intravenous infusion every 24 h to yield the area under the unbound concentration-time curve over 24 h ($fAUC_{24}$) of 64.4 mg·h/liter

(Table 1). A growth control was also included. With one exception, all control and drug-containing regimens were performed in two replicates. Syringe drivers were tested and flow rates through the CBR were calibrated prior to each study and monitored throughout to ensure that the system was performing optimally.

Samples for viable counting were collected at 0, 1, 2, 3, 5, 7, 24, 28, 48, 72, 96, and 120 h for planktonic bacteria (1 ml) and at 0, 3, 7, 24, 48, 72, 96, and 120 h for biofilm-embedded bacteria. For biofilm bacteria, a coupon holder containing three coupons was aseptically replaced with a blank holder at each time point. The removed coupons were rinsed twice in 10 ml of phosphate-buffered saline (PBS; pH 7.4) to remove planktonic cells and then placed in sterile tubes containing 10 ml PBS. Three alternating 1-min cycles of vortexing and sonication at 43 kHz followed by a final 1 min of vortexing were used to extract the biofilm-embedded cells (44). For the enumeration of the total bacterial population, 100 μl of appropriately diluted sample was manually plated onto drug-free CAMHA and incubated at 35°C for 48 h, due to the slow growth of the hypermutable CW8 isolate. The number of bacteria recovered from the coupons was expressed as the number of $\log_{10}$ CFU per square centimeter. Less-susceptible subpopulations were quantified for planktonic and biofilm bacteria at 0 (pretreatment), 24, 72, and 120 h following the start of treatment by plating 200 μl of appropriately diluted sample onto CAMHA (BD, Sparks, MD, USA) supplemented with meropenem at 6 mg/liter or 15 mg/liter or tobramycin at 3 mg/liter or 7.5 mg/liter. The plates were incubated for 48 h (meropenem) or 72 h (tobramycin) (15). MICs were determined at 0 and 120 h by the agar dilution method for colonies isolated from antibiotic-containing plates.

Pharmacokinetic validation. For antibiotic-containing regimens, 1-ml samples were collected in duplicate from the CBR at multiple time points across the duration of the study and immediately stored at −80°C. Meropenem and tobramycin in CAMHB–1% TSB were measured using validated liquid chromatography-tandem mass spectrometry assays (15). The protocol for measuring tobramycin and meropenem was similar to that described in our previous study (15), except for slight modifications required by the presence of TSB. Modifications included the gradient of the binary mobile phase, programmed as 0.25% formic acid (A)–acetonitrile (B) at 80:20 that changed over 0.5 min to A-B at 50:50, which was held for 2.51 min, followed by reequilibration to A-B at 80:20 for 4.59 min. The flow rate of the mobile phase was 0.3 ml/min, the column oven temperature was 30°C, and the total run time was 7 min. The lower limit of quantification was 0.10 mg/liter for meropenem and 0.50 mg/liter for tobramycin. The correlation coefficients for the calibration curve of meropenem (range, 0.10 to 50.0 mg/liter) and tobramycin (range, 0.50 to 25.0 mg/liter) were >0.998 and >0.999, respectively. The interday precisions were 1.1 to 5.8% for meropenem and 2.1 to 7.5% for tobramycin; interday accuracies were 96.3 to 106.9% for meropenem and 95.9 to 102.1% for tobramycin.

Pharmacodynamic analysis. Monotherapy or combination regimens causing a reduction of ≥1 $\log_{10}$ CFU/ml or CFU/cm² at a specified time relative to the baseline were considered active. Synergy was defined as ≥2 $\log_{10}$-CFU/ml or -CFU/cm² killing for the combination relative to that for the most active corresponding monotherapy at a specified time. Bacterial counts on antibiotic-containing plates were used to evaluate resistance emergence for different treatment regimens in comparison to the growth control. Mutation frequencies were calculated as the difference between the number of $\log_{10}$ CFU per milliliter (number of $\log_{10}$ CFU per square centimeter) on antibiotic-containing agar and the number of $\log_{10}$ CFU per milliliter (number of $\log_{10}$ CFU per square centimeter) on antibiotic-free agar at the same time point.

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Chapter 4

Clinically relevant epithelial lining fluid concentrations of meropenem with ciprofloxacin provide synergistic killing and resistance suppression of hypermutable *Pseudomonas aeruginosa* in a dynamic biofilm model

Preamble

Recurrent acute exacerbations of chronic respiratory infections in patients with CF often require long, repeated and aggressive antibacterial treatments to decrease the bacterial burden and restore the baseline lung function. In addressing **Aim 3**, we systematically investigated the impact of the most frequently used fluoroquinolone, ciprofloxacin, and meropenem as monotherapies and in combination against two hypermutable *P. aeruginosa* strains in the dynamic *in vitro* CBR over 120 h. This is the first study examining the effects of target site (lung fluid) concentrations for this combination against both modes of bacterial growth (planktonic, biofilm) under dynamic conditions. Meropenem was administered as a continuous infusion whereas ciprofloxacin was administered as short-term intermittent infusions representing FDA-approved daily dosing for intravenous administration. The findings from this Chapter supported **Hypothesis 3** that a combination regimen of ciprofloxacin with meropenem can overcome the emergence of ciprofloxacin-resistant subpopulations and achieve synergistic effects against hypermutable *P. aeruginosa* clinical and reference strains in the planktonic and biofilm state, with simulated lung fluid concentrations of each antibiotic. The results of this Chapter have been published in the ***Antimicrobial Agents and Chemotherapy***, as such they are presented in the format of a published article in the section immediately following. The results of this chapter

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PHARMACOLOGY

Clinically Relevant Epithelial Lining Fluid Concentrations of Meropenem with Ciprofloxacin Provide Synergistic Killing and Resistance Suppression of Hypermutable *Pseudomonas aeruginosa* in a Dynamic Biofilm Model

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ABSTRACT Treatment of exacerbations of chronic *Pseudomonas aeruginosa* infections in patients with cystic fibrosis (CF) is highly challenging due to hypermutability, biofilm formation, and an increased risk of resistance emergence. We evaluated the impact of ciprofloxacin and meropenem as monotherapy and in combination in the dynamic *in vitro* CDC biofilm reactor (CBR). Two hypermutable *P. aeruginosa* strains, PAOΔ*mutS* (MIC of ciprofloxacin [$MIC_{ciprofloxacin}$], 0.25 mg/liter; $MIC_{meropenem}$, 2 mg/liter) and CW44 ($MIC_{ciprofloxacin}$, 0.5 mg/liter; $MIC_{meropenem}$, 4 mg/liter), were investigated for 120 h. Concentration-time profiles achievable in epithelial lining fluid (ELF) following FDA-approved doses were simulated in the CBR. Treatments were ciprofloxacin at 0.4 g every 8 h as 1-h infusions (80% ELF penetration), meropenem at 6 g/day as a continuous infusion (CI) (30% and 60% ELF penetration), and their combinations. Counts of total and less-susceptible planktonic and biofilm bacteria and MICs were determined. Antibiotic concentrations were quantified by an ultrahigh-performance liquid chromatography photodiode array (UHPLC-PDA) assay. For both strains, all monotherapies failed, with substantial regrowth and resistance of planktonic (≥8 $\log_{10}$ CFU/ml) and biofilm (>8 $\log_{10}$ CFU/cm²) bacteria at 120 h ($MIC_{ciprofloxacin}$, up to 8 mg/liter; $MIC_{meropenem}$, up to 64 mg/liter). Both combination treatments demonstrated synergistic bacterial killing of planktonic and biofilm bacteria of both strains from ~48 h onwards and suppressed regrowth to ≤4 $\log_{10}$ CFU/ml and ≤6 $\log_{10}$ CFU/cm² at 120 h. Overall, both combination treatments suppressed the amplification of resistance of planktonic bacteria for both strains and of biofilm bacteria for CW44. The combination with meropenem at 60% ELF penetration also suppressed the amplification of resistance of biofilm bacteria for PAOΔ*mutS*. Thus, combination treatment demonstrated synergistic bacterial killing and resistance suppression against difficult-to-treat hypermutable *P. aeruginosa* strains.

KEYWORDS antibiotic resistance, biofilm infection, combination therapy, hypermutator

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TABLE 1 Clinically representative ELF concentrations, exposures, and pharmacokinetic/pharmacodynamic indices for ciprofloxacin and/or meropenem against PAOΔ*mutS* and CW44 in the CBR[a]

Isolate	Treatment	fC_{max}/fC_{min} or fC_{ss} (mg/liter)	$fAUC_{24}$ (mg · h/liter)	fC_{max}/MIC or fC_{ss}/MIC	$fT_{>MIC}$ (%)	$fAUC_{24}$/MIC
PAOΔ*mutS*	CIP at 0.4 g every 8 h	2.64/0.50	31.4	10.5		125.7
	MER at 6 g/day as CI	4.51	108	2.26	100	
	MER at 6 g/day as CI (60%)	9.02	216	4.51	100	
CW44	CIP at 0.4 g every 8 h	2.64/0.50	31.4	5.27		62.9
	MER at 6 g/day as CI	4.51	108	1.13	100	
	MER at 6 g/day as CI (60%)	9.02	216	2.25	100	

[a]The simulated half-life was 2.9 h for ciprofloxacin (CIP). The meropenem dosage regimen (6 g/day as a continuous infusion [CI]) was started at an unbound average steady-state concentration (fC_{ss}) of either 4.51 mg/liter (30% ELF penetration) or 9.02 mg/liter (60% ELF penetration). The simulated ELF penetration was 30% for meropenem (MER), unless specified as 60%, and was 80% for ciprofloxacin. fC_{max}, unbound peak concentration; fC_{min}, unbound minimum concentration before the next dose; $fAUC_{24}$, area under the unbound concentration-time curve over 24 h; fC_{max}/MIC, ratio of fC_{max} to MIC; $fT_{>MIC}$, cumulative percentage of a 24-h period that unbound concentrations exceeded the MIC; $fAUC_{24}$/MIC, ratio of $fAUC_{24}$ to MIC.

Respiratory tract infections in patients with cystic fibrosis (CF) present a serious medical challenge, and *Pseudomonas aeruginosa*, a difficult-to-treat pathogen, has a great impact on this group of patients (1). CF is a complex genetic disease caused by defective function of the CF transmembrane conductance regulator, resulting in altered sputum viscosity, disrupted airway anatomy, and impaired mucociliary clearance (2). These pathological conditions predispose to a repetitive cycle of acute infective exacerbations (AIEs) of chronic *P. aeruginosa* infections, causing worsened disease progression and increased early death in patients with CF (3, 4).

P. aeruginosa has the exceptional capacity to evade virtually all antimicrobials when used alone, resulting in treatment failure due to the selection of resistant mutants (5). Hypermutation (up to a 1,000-fold-increased mutation rate) occurs in up to ~65% of *P. aeruginosa* strains from patients with CF (6, 7) and is highly correlated with the establishment of chronic *P. aeruginosa* infection through biofilm formation (8). The important hallmark of biofilm-related infections is the increased secretion of the bacterial extracellular matrix, which limits the access of antimicrobials to the infecting pathogens and harbors phenotypic diversity (9). The presence of hypermutable *P. aeruginosa* in association with biofilm formation renders the treatment of AIE difficult and often results in persistence of infection and multidrug resistance (10, 11).

A combination of two or more antibiotics is currently recommended for the treatment of *P. aeruginosa* early exacerbations (12–14); however, information about the rational dosing of antibiotic combinations in CF is limited. Recently, we demonstrated that combining ciprofloxacin with meropenem, antibiotics with different mechanisms of action and resistance, combats hypermutable *P. aeruginosa* in the dynamic hollow-fiber infection model (HFIM) (15). However, the antibacterial activity of this combination against hypermutable *P. aeruginosa* embedded in biofilms has not been explored. Therefore, the dynamic CDC biofilm reactor (CBR) model was used in the present study. The CBR is a well-accepted, state-of-the-art pharmacokinetic/pharmacodynamic (PK/PD) model that allows the simulation of clinically relevant epithelial lining fluid (ELF) concentration-time profiles as seen in patients and also enables the examination of antibacterial effects on both planktonic and biofilm bacteria simultaneously.

The main objective of the present study was to systemically evaluate the impact of ciprofloxacin and meropenem as monotherapy and in combination against hypermutable *P. aeruginosa* strains in the dynamic *in vitro* CBR model. We examined the time course of bacterial killing and resistance suppression of both planktonic and biofilm bacteria over 120 h by simulating clinically relevant ELF concentrations of ciprofloxacin and meropenem alone and in combination in the CBR.

RESULTS

PK validation and microbiological response. The observed ciprofloxacin and meropenem concentrations in the CBR were on average within 5% of the targeted concentrations (Table 1). Viable-cell-count profiles for total populations of planktonic

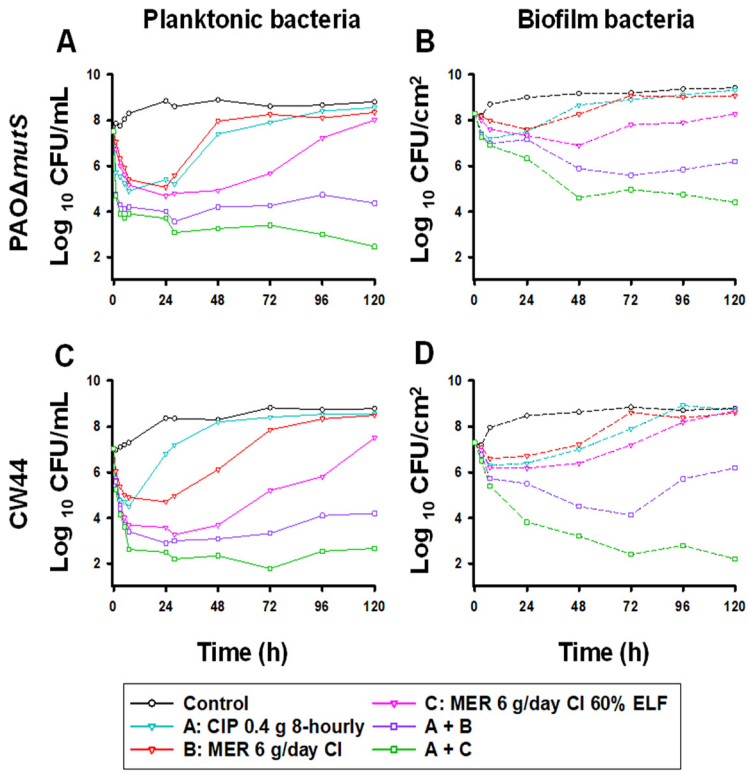

FIG 1 Total viable counts for growth controls and treatments with ciprofloxacin (CIP) and/or meropenem (MER) with clinically relevant ELF concentration-time profiles. The simulated ELF penetration was 30% for meropenem, unless specified as 60%, and was 80% for ciprofloxacin. Samples were obtained from the medium within the reactor, i.e., planktonic bacteria, and from coupons, i.e., biofilm bacteria. The *y* axis starts from the limit of counting.

and biofilm bacteria ($n = 2$ measurements) for both strains are shown in Fig. 1, while the corresponding profiles for less-susceptible populations of PAOΔ*mutS* and CW44 are shown in Fig. 2 and 3, respectively. Log changes in viable-cell counts of total bacteria, mutant frequencies, and MICs are shown in Tables 2 to 4, respectively.

Planktonic bacteria. The starting inocula (means ± standard deviations [SD]) in all arms were 7.5 ± 0.18 $\log_{10}$ CFU/ml ($n = 6$) for PAOΔ*mutS* and 7.0 ± 0.094 $\log_{10}$ CFU/ml ($n = 6$) for CW44. PAOΔ*mutS* grew rapidly in growth control chambers and plateaued at ~8.8 $\log_{10}$ CFU/ml by 24 h (Fig. 1A). Less-susceptible populations plateaued at ~5.7 and 5.2 $\log_{10}$ CFU/ml on agar containing 0.57 mg/liter and 1.25 mg/liter of ciprofloxacin and grew to ~3.4 and 1.9 $\log_{10}$ CFU/ml on agar containing 5 mg/liter and 10 mg/liter of meropenem at 120 h, respectively (Fig. 2A, C, E, and G). CW44 grew to ~8.3 $\log_{10}$ CFU/ml by 24 h and plateaued at ~8.7 $\log_{10}$ CFU/ml from 72 h (Fig. 1C). Less-susceptible populations increased approximately in proportion to the growth of the total bacterial population (Fig. 3A, C, E, and G and Table 3).

With PAOΔ*mutS*, ciprofloxacin monotherapy produced an initial killing of ~2.6 $\log_{10}$ CFU/ml at 7 h, followed by slow regrowth close to the values of the growth control by 96 h (Fig. 1A and Table 2). Amplification of ciprofloxacin-less-susceptible populations was observed, such that a large proportion of the entire population grew on plates containing 0.57 mg/liter and 1.25 mg/liter of ciprofloxacin at 120 h (Fig. 2A and C and Table 3). Emergence of resistance was observed with an ~3-log increase of the ciprofloxacin-resistant population compared to the growth control at 120 h. The MIC of the colonies isolated from agar plates containing 1.25 mg/liter of ciprofloxacin was 8 mg/liter at 120 h (Table 4). For CW44, ciprofloxacin monotherapy achieved an initial killing of ~1.8 $\log_{10}$ CFU/ml at 7 h, followed by steady regrowth approaching control values by 48 h (Fig. 1C and Table 2). Growth of ~8.1 $\log_{10}$ CFU/ml on agar plates containing

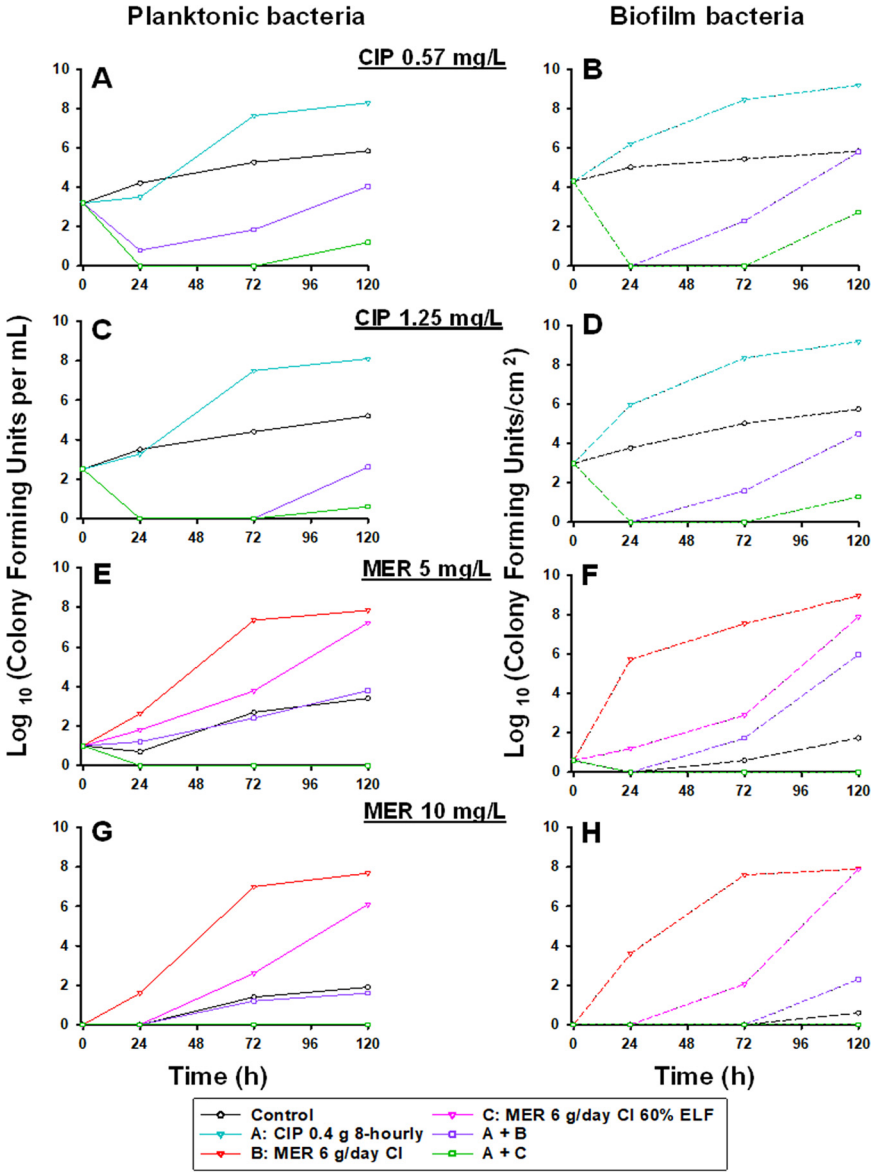

FIG 2 Effect of each dosage regimen on the counts of PAOΔ*mutS* bacteria able to grow on agar plates containing 0.57 or 1.25 mg/liter of ciprofloxacin or 5 or 10 mg/liter of meropenem. To differentiate less-susceptible subpopulations from the predominant population, the antibiotic concentrations in agar were based upon Etest MICs, which were 0.50 mg/liter for meropenem and 0.064 mg/liter for ciprofloxacin (19).

0.57 mg/liter and 1.25 mg/liter of ciprofloxacin was obtained at 120 h (Fig. 3A and C). Emergence of resistance was observed with a >4-log increase of ciprofloxacin-resistant bacteria at 120 h on both sets of drug plates compared to the growth control. The MIC at 120 h was 8 mg/liter (Table 4).

For PAOΔ*mutS*, meropenem monotherapy representing 30% ELF penetration produced ~2.4-$\log_{10}$ CFU/ml bacterial killing at 24 h, followed by substantial regrowth close to the values for the growth control; with 60% ELF penetration, an additional ~0.5-$\log_{10}$ CFU/ml killing was achieved at 24 h (Fig. 1A and Table 2). Less-susceptible populations of ≤7.8 $\log_{10}$ CFU/ml on plates containing 5 mg/liter of meropenem and of ≤7.6 $\log_{10}$ CFU/ml on plates containing 10 mg/liter of meropenem were obtained for both levels of ELF penetration (Fig. 2E and G). Emergence of resistance was observed with both meropenem monotherapies, with up to a 5.7-log CFU/ml increase of the meropenem-resistant bacteria compared to the growth control at 120 h. The MICs of

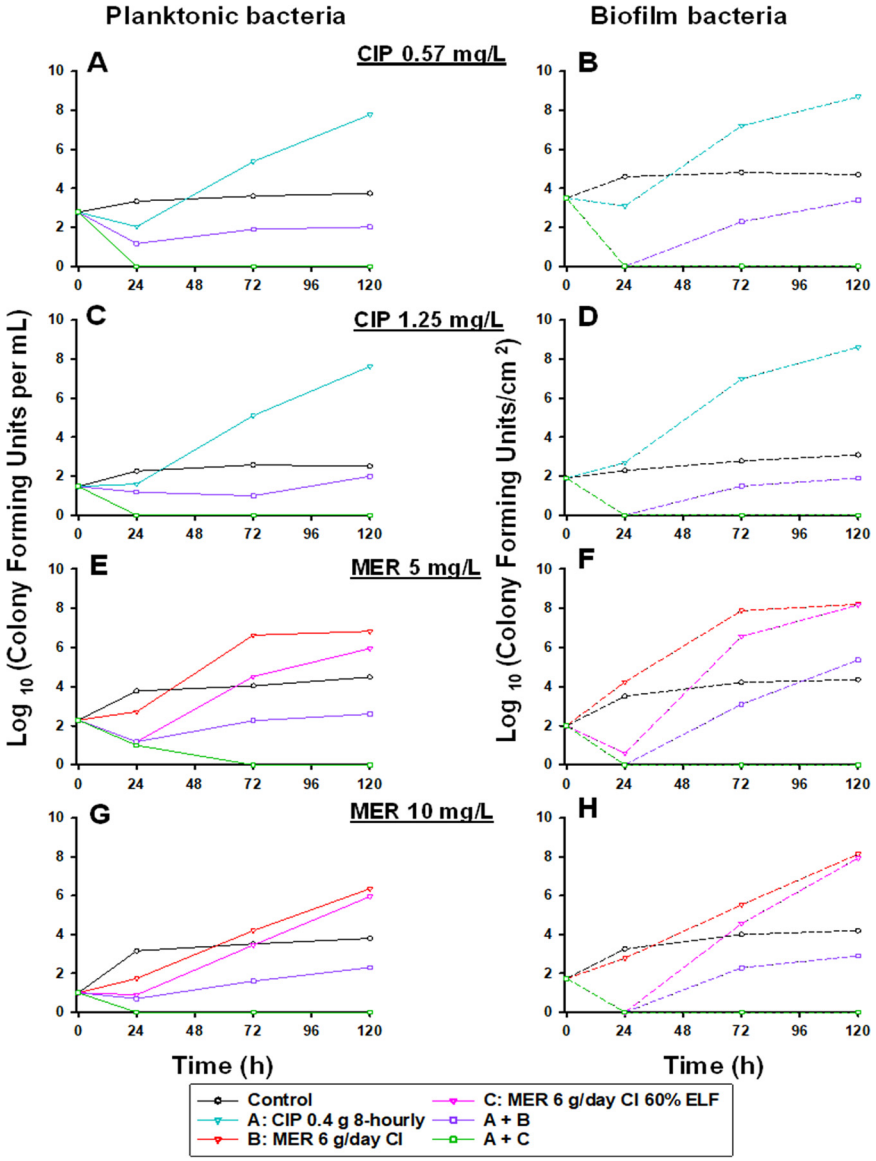

FIG 3 Effect of each dosage regimen on the CW44 counts able to grow on agar plates containing 5 or 10 mg/liter of meropenem or 0.57 or 1.25 mg/liter of ciprofloxacin. To differentiate less-susceptible subpopulations from the predominant population, the antibiotic concentrations in agar were based upon Etest MICs, which were 0.5 mg/liter for meropenem and 0.19 mg/liter for ciprofloxacin (19).

colonies recovered from plates containing 10 mg/liter meropenem were 64 mg/liter for 30% ELF penetration and 32 mg/liter for 60% ELF penetration at 120 h (Table 4). For CW44, meropenem monotherapy simulating 30% ELF penetration produced ~2.3 $\log_{10}$ CFU/ml over the first 24 h, followed by regrowth close to the value for the growth control at 120 h, whereas with meropenem at 60% ELF penetration, ~1.2 $\log_{10}$ CFU/ml more killing was achieved at 24 h, and regrowth stayed ~1 log below the control values (Fig. 3C and Table 2). Emergence of meropenem resistance was observed for both levels of ELF penetration in comparison to the growth control at 120 h (Fig. 3E and G and Table 3). The MICs were 128 mg/liter for 30% ELF penetration and 64 mg/liter for 60% ELF penetration at 120 h (Table 4).

For PAOΔ*mutS*, the combination of ciprofloxacin with meropenem representing 30% ELF penetration produced rapid initial killing of ~3.2 $\log_{10}$ CFU/ml at 3 h, which increased to ~3.9 $\log_{10}$ CFU/ml by 28 h; the combination simulating meropenem at 60% ELF penetration achieved an additional ~0.5-$\log_{10}$ CFU/ml killing at 28 h (Fig. 1A

TABLE 2 Log changes in viable-cell counts of total bacteria at various time points with clinically relevant ELF concentration exposures of meropenem and/or ciprofloxacin[a]

Isolate	Time (h)	Log change= [$\log_{10}(CFU_t) - \log_{10}(CFU_0)$]									
		A: CIP 0.4 g 8-hourly		B: MER 6 g/day CI		C: MER 6 g/day CI 60% ELF		A + B		A + C	
		Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria
PAOΔ*mutS*	1	-1.80		-0.44		-0.79		-2.70		-2.82	
	3	-1.98	-0.86	-1.19	-0.12	-1.49	-0.30	-3.21	-0.96	-3.61	-1.03
	5	-2.28		-1.57		-1.90		-3.41		-3.81	
	7	-2.60	-0.95	-2.11	-0.32	-2.34	-0.70	-3.36	-1.32	-3.61	-1.40
	24	-2.11	-0.80	-2.40	-0.70	-2.90	-0.97	-3.51	-1.14	-3.83	-1.97
	28	-2.31		-1.93		-2.71		-3.95		-4.43	
	48	-0.11	0.37	0.45	-0.03	-2.61	-1.60	-3.31	-2.42	-4.27	-3.70
	72	0.39	0.60	0.75	0.79	-1.66	-0.50	-3.25	-2.71	-4.16	-3.34
	96	0.89	0.81	0.59	0.72	-0.28	-0.40	-2.78	-2.46	-4.52	-3.56
	120	1.05	1.02	0.83	0.78	0.50	-0.02	-3.14	-2.11	-5.05	-3.90
CW44	1	-1.33		-0.98		-1.35		-1.45		-1.78	
	3	-1.55	-0.42	-1.65	-0.20	-2.47	-0.35	-2.62	-0.53	-2.88	-0.80
	5	-1.62		-2.03		-3.01		-3.31		-3.43	
	7	-1.80	-0.65	-2.13	-0.70	-3.35	-0.85	-3.63	-1.58	-4.41	-1.90
	24	-0.23	-0.90	-2.33	-0.59	-3.45	-0.98	-4.13	-1.80	-4.54	-3.49
	28	0.17		-2.06		-3.77		-4.03		-4.83	
	48	1.17	0.30	-0.91	-0.09	-3.35	-0.90	-3.95	-2.80	-4.69	-4.10
	72	1.38	0.85	0.83	1.33	-1.83	0.10	-3.41	-1.98	-5.15	-4.90
	96	1.51	1.63	1.31	1.08	-1.22	0.90	-2.93	-1.59	-4.49	-4.51
	120	1.53	1.43	1.47	1.30	0.48	1.40	-1.83	-1.11	-4.37	-5.28

[a]The green background indicates synergy (a ≥2-$\log_{10}$ decrease in the CFU per milliliter or CFU per square centimeter with the combination compared to its most active component) (for planktonic bacteria, a ≥2-$\log_{10}$ decrease in the CFU per milliliter compared to the initial inoculum was also required for synergy); the blue background indicates a 1.0- to <2-$\log_{10}$ decrease in the number of CFU per milliliter or CFU per square centimeter with the combination compared to its most active component. MER, meropenem; CIP, ciprofloxacin.

and Table 2). With both levels of meropenem ELF penetration, enhanced killing by the combination (reduction of ≥1 to <2 $\log_{10}$ CFU/ml compared to the most active corresponding monotherapy) was observed from the first few hours, and synergy occurred from 48 h and 72 h for the low and high levels of meropenem ELF penetration, respectively (Table 2). Growth on agar plates containing ciprofloxacin at 1.25 mg/liter was ~2.6 $\log_{10}$ CFU/ml at 120 h, growth on those containing 10 mg/liter meropenem was ~1.6 $\log_{10}$ CFU/ml for 30% ELF penetration (Fig. 2C and G), and the MIC was 4 mg/liter for ciprofloxacin and 16 mg/liter for meropenem at 120 h (Table 4). With the combination simulating meropenem at 60% ELF penetration, at 120 h, ~0.6 $\log_{10}$ CFU/ml were observed on agar containing 1.25 mg/liter of ciprofloxacin, but no colonies were present on meropenem-containing agar.

For CW44, bacterial killing of ≥4 $\log_{10}$ CFU/ml was achieved with the combination for both meropenem ELF penetration levels (Fig. 1C and Table 2), with greater killing for the regimen representing 60% ELF penetration. Enhanced bacterial killing by the combination compared with the most active monotherapy occurred within the first day, and synergy was observed from 48 h and 72 h, respectively (Table 2). Growth of ~2.3 $\log_{10}$ CFU/ml was obtained on plates containing 10 mg/liter meropenem with the combination simulating 30% ELF penetration, which was ~1.5 $\log_{10}$ CFU/ml below the control values; no colonies were detected from 24 h onwards with the combination simulating 60% ELF penetration (Fig. 3G). No ciprofloxacin-resistant colonies were observed with the combination simulating meropenem at 60% ELF penetration. For the combination with meropenem at 30% ELF penetration, ~2 $\log_{10}$ CFU/ml were retrieved from plates containing 1.25 mg/liter ciprofloxacin, which was ~0.5 $\log_{10}$ CFU/ml below the growth control counts (Fig. 3A and C and Table 3). For this combination regimen, at 120 h, the MICs were 4 mg/liter for ciprofloxacin and 32 mg/liter for meropenem (Table 4).

Biofilm-embedded bacteria. The starting inocula (means ± SD) in all arms were 8.3 ± 0.12 $\log_{10}$ CFU/cm² ($n = 2$ coupons × 6) and 7.3 ± 0.07 $\log_{10}$ CFU/cm² ($n = 2 \times 6$) for PAOΔ*mutS* and CW44, respectively. By 24 h, the growth controls for both isolates grew to ~9 $\log_{10}$ CFU/cm² and ~8.4 $\log_{10}$ CFU/cm², respectively, and pla-

TABLE 3 Log_{10} mutant frequencies at 5 mg/liter and 10 mg/liter meropenem and at 0.57 mg/liter and 1.25 mg/liter ciprofloxacin[a]

			CIPROFLOXACIN				MEROPENEM			
			0.57 mg/L		1.25 mg/L		5 mg/L		10 mg/L	
Isolate	Time (h)	Arm	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria
PAOΔ*mutS*	0	All Regimens	-4.00	-4.31	-5.01	-5.30	-6.51	-7.70	-7.51	-8.30
	24	Control	-4.39	-4.63	-4.95	-5.22	-7.15	-8.00	-8.85	-9.00
		A: CIP 0.4 g 8-hourly	-1.90	-1.29	-2.13	-1.53				
		B: MER 6g/d CI					-2.43	-1.86	-3.46	-4.00
		C: MER 6g/d CI 60% ELF					-2.91	-6.13	-4.71	-7.33
		A + B	-3.20	-7.16	-4.00	-7.16	-2.80	-7.16	-4.00	-7.16
		A + C	-3.70	-6.33	-3.70	-6.33	-3.70	-6.33	-3.70	-6.33
	72	Control	-3.33	-3.75	-4.17	-4.21	-5.91	-7.94	-7.21	-9.20
		A: CIP 0.4 g 8-hourly	-0.44	0.25	-0.55	-0.40				
		B: MER 6g/d CI					-1.89	-1.53	-1.26	-1.49
		C: MER 6g/d CI 60% ELF					-2.07	-4.90	-3.24	-5.75
		A + B	-2.41	-3.30	-2.86	-3.99	-1.86	-3.85	-3.06	-5.59
		A + C	-3.35	-4.96	-3.35	-4.96	-3.35	-4.96	-3.35	-4.96
	120	Control	-3.11	-3.52	-3.67	-3.60	-5.41	-7.68	-6.91	-8.83
		A: CIP 0.4 g 8-hourly	-0.26	-0.12	-0.46	-0.22				
		B: MER 6g/d CI					-0.35	-0.12	-0.66	-1.18
		C: MER 6g/d CI 60% ELF					-0.43	-0.63	-1.40	-1.60
		A + B	-0.32	-0.45	-1.77	-2.99	-0.21	-0.57	-2.77	-3.89
		A + C	-1.86	-1.66	-1.26	-3.10	-2.46	-4.40	-2.46	-4.40
CW44	0	All Regimens	-4.25	-3.79	-5.55	-5.40	-4.74	-5.30	-5.53	-6.30
	24	Control	-5.02	-3.87	-6.19	-6.10	-4.59	-4.97	-5.21	-5.32
		A: CIP 0.4 g 8-hourly	-4.76	-3.30	-5.20	-3.70				
		B: MER 6g/d CI					-1.98	-2.48	-2.96	-3.93
		C: MER 6g/d CI 60% ELF					-2.40	-5.58	-2.68	-6.18
		A + B	-1.72	-5.50	-1.71	-5.50	-1.72	-5.50	-2.20	-5.50
		A + C	-2.49	-3.81	-2.49	-3.81	-1.49	-3.81	-2.49	-3.81
	72	Control	-5.23	-4.03	-6.25	-6.07	-4.63	-4.79	-5.31	-5.33
		A: CIP 0.4 g 8-hourly	-3.03	-0.69	-3.30	-0.91				
		B: MER 6g/d CI					-1.23	-0.74	-3.66	-3.10
		C: MER 6g/d CI 60% ELF					-0.68	-0.63	-2.75	-2.64
		A + B	-1.42	-1.83	-2.32	-2.62	-1.06	-1.02	-1.72	-1.83
		A + C	-1.78	-2.40	-1.78	-2.40	-1.78	-2.40	-1.78	-2.40
	120	Control	-5.04	-4.09	-6.28	-5.70	-4.30	-4.44	-4.99	-5.00
		A: CIP 0.4 g 8-hourly	-0.46	-0.03	-0.93	-0.11				
		B: MER 6g/d CI					-1.67	-0.39	-0.64	-0.46
		C: MER 6g/d CI 60% ELF					-1.55	-0.52	-1.31	-0.76
		A + B	-1.99	-2.80	-2.20	-4.29	-1.60	-0.82	-1.90	-3.29
		A + C	-2.66	-2.20	-2.66	-2.20	-2.66	-2.20	-2.66	-2.20

[a]The red background indicates a high mutant frequency, i.e., a large proportion of less-susceptible bacteria being present in the total population; the green background indicates a low mutant frequency, i.e., a small proportion of less-susceptible bacteria being present in the total population. CIP, ciprofloxacin; MER, meropenem.

teaued until 120 h (Fig. 1B and D). Less-susceptible populations for ciprofloxacin (0.57 and 1.25 mg/liter on agar) and meropenem (5 and 10 mg/liter on agar) grew to ~1.7 and 5.5 log_{10} CFU/cm^2, respectively, at 120 h (Fig. 2 and Fig. 3B, D, F, and H).

With PAOΔ*mutS*, the monotherapy treatments produced ≤0.9-log_{10} CFU/cm^2 killing at 7 h, followed by regrowth close to the growth control; with meropenem at 60% ELF penetration, ~1.6-log_{10} CFU/cm^2 bacterial killing was achieved at 48 h, and regrowth remained ~1 log_{10} CFU/cm^2 below the control values at 120 h (Fig. 1B and Table 2). Substantial increases in less-susceptible populations occurred with ciprofloxacin treatment and both levels of ELF penetration of meropenem (Fig. 2B, D, F, and H). At 120 h,

TABLE 4 MIC values for colonies retrieved from antibiotic-containing agar plates (1.25 mg/liter ciprofloxacin and 10 mg/liter meropenem) at 0 and 120 h for each dosage regimen[a]

Isolate	Arm	Time (h)	MIC (mg/liter)			
			Ciprofloxacin at 1.25 mg/liter		Meropenem at 10 mg/liter	
			Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria
PAOΔ*mutS*	Control	0	2	2	NC	NC
		120	4	2	4	NC
	CIP at 0.4 g every 8 h	120	8	4	–	–
	MER at 6 g/day as CI	120	–	–	64	16
	MER at 6 g/day as CI (60% ELF)	120	–	–	32	16
	MER at 6 g/day as CI + CIP at 0.4 g every 8 h	120	4	2	16	8
	MER at 6 g/day as CI (60% ELF) + CIP at 0.4 g every 8 h	120	NC	NC	NC	NC
CW44	Control	0	2	2	8	4
		120	4	4	16	16
	CIP at 0.4 g every 8 h	120	8	8	–	–
	MER at 6 g/day as CI	120	–	–	128	64
	MER at 6 g/day as CI (60% ELF)	120	–	–	64	32
	MER at 6 g/day as CI + CIP at 0.4 g every 8 h	120	4	2	32	8
	MER at 6 g/day as CI (60% ELF) + CIP at 0.4 g every 8 h	120	NC	NC	NC	NC

[a]NC, no colonies grew on the antibiotic-containing plates; –, not tested.

the MIC was 4 mg/liter for ciprofloxacin; the MIC for meropenem at 30% and 60% ELF penetration was 16 mg/liter (Table 4). In contrast, ciprofloxacin in combination with meropenem produced enhanced bacterial killing, with regrowth suppressed from 48 to 120 h. Growth plateaued at ~6 $\log_{10}$ CFU/cm^2, which was ~3.5 $\log_{10}$ CFU/cm^2 below the control values. The combinations with both levels of meropenem ELF penetration were synergistic from 48 h onwards (Fig. 1B and Table 2). After 120 h of treatment with combinations containing meropenem at either level of ELF penetration, growth on agar containing meropenem at 10 mg/liter or ciprofloxacin at 1.25 mg/liter was >5 $\log_{10}$ units lower than that with the corresponding monotherapy (Fig. 2B, D, F, and H). At 120 h, with the combination containing the lower level of meropenem ELF penetration, the MIC of ciprofloxacin (MIC_{CIP}) was 2 mg/liter, and that of meropenem was 8 mg/liter (Table 4). A negligible number (~0.6 $\log_{10}$ CFU/cm^2) of colonies were retrieved from plates containing ciprofloxacin at 1.25 mg/liter, and no colonies were observed on agar containing meropenem at 5 or 10 mg/liter (Fig. 2B, D, F, and H).

With CW44, all monotherapies produced bacterial killing that closely matched that of PAOΔ*mutS* at 24 h, followed by slow regrowth to values close to those of the growth control by 72 to 120 h (Fig. 1D and Table 2). Substantial increases in less-susceptible populations for both ciprofloxacin (0.57 and 1.25 mg/liter on agar) and meropenem (5 and 10 mg/liter on agar) were observed (Fig. 3B, D, F, and H and Table 3). Emergence of resistance was observed with a ~3.5- to 6-$\log_{10}$ CFU/cm^2 increase of the ciprofloxacin- and meropenem-resistant bacteria compared to the growth control. MICs at 120 h were 8 mg/liter for ciprofloxacin, 64 mg/liter for meropenem at 30% ELF penetration, and 32 mg/liter for meropenem at 60% ELF penetration (Table 4). The combinations simulating meropenem at 30% or 60% ELF penetration achieved a substantially greater antibacterial effect. With meropenem at 30% ELF penetration, synergy was observed from 48 h onwards, and with meropenem at 60% ELF penetration, an increasing level of synergy occurred from 24 h; regrowth remained suppressed below ~6 $\log_{10}$ CFU/cm^2 at 120 h (Fig. 1D and Table 2). Less-susceptible populations for both ciprofloxacin and meropenem were observed at 120 h; these were ~1 to 2 $\log_{10}$ CFU/cm^2 below the control values, except on agar containing 5 mg/liter meropenem, where they were slightly above the values of the growth control (Fig. 3B, D, F, and H and Table 3). At the end of treatment with the combination containing meropenem at 30% ELF penetration, the MIC of ciprofloxacin was 2 mg/liter, and that of meropenem was 8 mg/liter at this time point; no colonies were observed on antibiotic-

containing agar plates with the combination with meropenem at 60% ELF penetration (Table 4).

DISCUSSION

Ciprofloxacin and meropenem have been widely used to treat respiratory infections caused by *P. aeruginosa*, including in patients with CF (16, 17). However, *P. aeruginosa* can readily acquire resistance to these antibiotics in monotherapy via selection of mutations (18). Rates of resistance to these antibiotics of >30% have been reported for isolates from respiratory infections in patients with CF, with hypermutable strains especially having high resistance rates (19, 20). Current guidelines endorse the use of antipseudomonal agents in combination for the treatment of acute exacerbations of chronic respiratory infections in patients with CF (12–14). However, the antibacterial effect of ciprofloxacin with meropenem against *P. aeruginosa* isolates has never been explored in a dynamic biofilm model. This study systematically investigated the impact of ciprofloxacin and meropenem, in monotherapies and in combination, on bacterial killing and resistance emergence of hypermutable *P. aeruginosa* isolates in the CBR. The simulated pharmacokinetic profiles were representative of antibiotic concentrations expected to be achieved in the ELF of patients with CF following intravenous (i.v.) administration of approved daily doses.

For quinolones such as ciprofloxacin, antibacterial activity has been correlated with the ratio of the unbound (free) area under the plasma concentration-time curve (*f*AUC) to the MIC (*f*AUC/MIC) and the ratio of the free plasma peak concentration (fC_{max}) to the MIC (fC_{max}/MIC) (21–23). In acutely ill patients with bacterial infections, an AUC/MIC of 125 (corresponding to an *f*AUC/MIC of 87.5) and a C_{max}/MIC of ≥8 (fC_{max} of ≥5.6) have been proposed for clinical cure (24–26). However, these targets were often not reached in hospitalized patients infected with a strain having an MIC of ≥0.5 mg/liter (27). It is very likely that the isolates used in establishing the above-mentioned targets were not hypermutable. In the present study, the corresponding values for *f*AUC/MIC and fC_{max}/MIC were 125.7 and 10.5 for PAOΔ*mutS* (MIC_{CIP} = 0.25 mg/liter) and 62.9 and 5.27 for CW44 (MIC_{CIP} = 0.5 mg/liter) (Table 1), exceeding both PK/PD targets described above for PAOΔ*mutS* but not for CW44. However, suppression of regrowth and resistance was not achieved with ciprofloxacin monotherapy against planktonic and biofilm bacteria of either strain. This result was in agreement with our previous HFIM study where ciprofloxacin monotherapy was ineffective in suppressing the regrowth of planktonic CW44 (15). Similarly, in another HFIM study, extensive emergence of resistance was observed after 48 h with a regimen generating a ciprofloxacin AUC/MIC of 180 against *P. aeruginosa* strains in planktonic growth, although it was not stated whether the isolates were hypermutable (28). The emergence of resistance in both studies (15, 28) was consistent with the inverted U relationship and mutant selection window (29, 30). In the present study, the effect of ciprofloxacin monotherapy on biofilm bacteria was attenuated, compared to that on planktonic bacteria. There are two factors that may have contributed to the poorer effect against biofilm bacteria. First, although quinolones are considered to diffuse readily through the biofilm matrix, low oxygen concentrations within the biofilm decrease their antibacterial effect due to the insufficient formation of reactive oxygen species (31, 32). Second, following exposure to a subinhibitory ciprofloxacin concentration in a static system, mutations in certain efflux pump regulators were more frequently found in biofilm growth than in planktonic bacteria (33).

The antibacterial activity of β-lactams, including meropenem, has been traditionally correlated with the fraction of the dosing interval for which the unbound concentration remains above some multiple of the MIC of the infecting pathogen ($fT_{>MIC}$) (21–23, 34). Traditionally, an $fT_{>MIC}$ of ~40% was considered to be necessary for the optimal bactericidal activity of meropenem (21, 34). More recent studies suggested a value of 100% $fT_{>4-5\times\ MIC}$ to be necessary for resistance suppression (25). In our previous HFIM studies, meropenem monotherapy administered as intermittent (short or prolonged) infusions at a daily dose of 3 g or 6 g or as a continuous infusion (CI) at 3 g/day was

ineffective in suppressing the regrowth of planktonic PAOΔ*mutS* (35) and CW44 (15), for $fT_{>MIC}$ values of 61% and 69 to 88%, and extensive emergence of resistance occurred. In the present study, in the CBR model, 6 g/day meropenem was delivered as a CI to maximize $fT_{>MIC}$. Since different levels of ELF penetration of meropenem have been reported in the literature, two ELF penetration levels (30% and 60%) were examined (36–39). When meropenem was administered as monotherapy at both levels of ELF penetration, the concentrations remained above the MIC at all times. Indeed, for PAOΔ*mutS*, the unbound meropenem concentrations remained at >2× MIC and >4× MIC across the entire study duration with ELF penetrations of 30% and 60%, respectively; the corresponding values for CW44 were >1× MIC and >2× MIC. Nevertheless, while some bacterial killing was observed initially with planktonic bacteria and to a lesser extent with biofilm bacteria, extensive regrowth occurred, which was associated with the amplification of meropenem-resistant cells. This failure of meropenem CI, representing an extreme mode of administration, raises questions about the use of monotherapy, especially against hypermutable strains, because such strains can readily develop resistance and become multidrug resistant (MDR) due to the amplification of resistant mutant subpopulations (40).

Combination therapy could be a viable option for the treatment of *P. aeruginosa* infections involving hypermutable strains. Increases in minimum bactericidal concentrations observed with single agents can be minimized by combining two antipseudomonal agents (40). In the present study, we considered not only synergy but also enhanced bacterial killing (reduction of ≥1 to <2 $\log_{10}$ CFU compared to the most active monotherapy), since even a relatively small increase in activity with combination therapy may be beneficial for patient care. In the CBR studies with combination regimens containing meropenem at either level of penetration against both PAOΔ*mutS* and CW44, enhanced activity was evident from the early stages of treatment, particularly for planktonic bacteria, and synergy against biofilm bacteria occurred across the last 3 days. The synergy observed was notable, given that the isolates are strong hypermutators.

The enhanced and synergistic bacterial killing observed with the combination regimens in the present study may be due to differences in the mechanism of action and resistance of each antibiotic. Combining antibiotics with different mechanisms requires separate and independent mutations for resistance development and may help to minimize the chances of positive selection of resistant mutants (40). Resistance to ciprofloxacin occurs primarily via target-site mutation and overexpression of efflux pumps, whereas resistance mechanisms against meropenem include enzymatic inactivation via carbapenemases, AmpC β-lactamase overexpression, and reduced expression of the gene for the outer membrane porin OprD, which decreases access to the periplasmic space and the penicillin-binding proteins (PBPs) located there (41, 42). In addition, ciprofloxacin has been shown to increase the permeability of the outer membrane of *P. aeruginosa* and thereby may increase meropenem concentrations in the periplasm (43). The differences in the mechanism of action and resistance of each antibiotic in the combination, together with possibly higher meropenem concentrations in the periplasm, may have contributed to the enhanced and synergistic bacterial killing and resistance suppression against hypermutable strains in the present study. Moreover, strain CW44 was particularly challenging since, in addition to the mutator phenotype, it already contained relevant meropenem resistance mechanisms, including OprD inactivation, PBP3 modification, and AmpC overexpression (19). This study suggests that the above-mentioned types of mechanistic synergy may apply not only to planktonic bacteria but also to biofilm bacteria, although given the complex nature of biofilm matrices, other mechanisms may also be operative for this growth form.

To our knowledge, this is the first study examining the antibacterial activity of meropenem and ciprofloxacin in combination against hypermutable *P. aeruginosa* isolates in planktonic and biofilm growth in a dynamic biofilm model. Previously, we evaluated this combination in an HFIM study simulating ELF pharmacokinetics (15). However, that study employed only one clinical isolate (CW44) and investigated only

planktonic growth. In 72-h static-concentration time-kill (SCTK) studies, we previously explored the activity of this combination against a range of *P. aeruginosa* isolates, but the use of static concentrations and the examination of only planktonic growth were limitations (15). Some other studies also investigated this combination *in vitro* via checkboard methods and 24-h SCTK studies against *P. aeruginosa* isolates from different patient groups and reported synergistic outcomes (44–47). However, those studies did not quantify the time course of bacterial killing in a biofilm, nor did they examine the emergence of resistance. Importantly, the latter studies did not employ a dynamic system, nor did they include hypermutable isolates, and many of the antibiotic concentrations in the SCTK study were higher (reflecting those in plasma) than those that are clinically achievable in ELF after intravenous exposure.

The present study has a number of particular strengths. First, this is the only study to examine the impact of the ciprofloxacin-meropenem combination against biofilm growth and newly shed bacteria from a biofilm. Second, this is the only *in vitro* biofilm study exploring the effects of concentration-time profiles representative of those in ELF for this combination against both modes of growth of hypermutable *P. aeruginosa*. To encompass different levels of meropenem ELF exposure, we examined both 30% and 60% penetration, reflecting literature reports (36–39). Both planktonic and biofilm bacteria were enumerated over 5 days, and emergence of resistance was examined. In addition, two hypermutable *P. aeruginosa* isolates (1 reference and 1 clinical) were employed to inform future studies. Conversely, this study has some limitations. In the future, remaining questions need to be addressed. First, genomic analysis of emergent resistant populations would assist in the confirmation of mechanisms involved in adaptation and resistance amplification, which commonly occur in hypermutable isolates. Furthermore, confocal imaging would elucidate changes in biofilm structure. This information will assist in developing next-generation mechanism-based mathematical models to explore antibiotic effects on biofilm bacteria. In addition, although the CBR is an ideal *in vitro* dynamic model for examining antibacterial effects, it does not allow the assessment of immune system effects on residual populations, and therefore, *in vivo* studies are required.

In summary, this study showed that neither ciprofloxacin nor meropenem was effective as monotherapy against planktonic and biofilm bacteria of hypermutable *P. aeruginosa* strains. However, both antibiotics in combination regimens demonstrated promising results when simulating ELF pharmacokinetic profiles achievable with FDA-approved daily doses in patients with CF. The combination regimens exhibited enhanced bacterial killing and resistance suppression against both isolates. Thus, this promising combination warrants further evaluation.

MATERIALS AND METHODS

Antibiotics, media, bacterial isolates, and susceptibility testing. Stock solutions of ciprofloxacin (Sigma-Aldrich, Sydney, Australia) and meropenem (Kabi, Melbourne, Australia) were prepared in Milli-Q water as described previously (15). All experiments used cation-adjusted Mueller-Hinton broth (CAMHB) with 1% tryptic soy broth (TSB; BD, Sparks, MD, USA) containing 25 mg/liter Ca^{2+} and 12.5 mg/liter Mg^{2+}. Viable-cell counting was carried out on cation-adjusted Mueller-Hinton agar (CAMHA; BD, Sparks, MD, USA). Drug-containing agar plates were prepared on the day of the experiment by adding appropriate volumes of antibiotic stock solutions to CAMHA.

The hypermutable *P. aeruginosa* strain PAOΔ*mutS* and a clinical hypermutable *P. aeruginosa* isolate (CW44) were examined. PAOΔ*mutS* is the isogenic hypermutable strain of the *P. aeruginosa* wild-type reference strain PAO1, constructed by Mena et al. via *mutS* gene deletion (48). Clinical isolate CW44 was obtained from an adult patient with CF and a respiratory infection and was documented to be deficient in *mutL* (19). MICs for PAOΔ*mutS* and CW44 were determined in triplicate on each of three separate days using agar dilution according to Clinical and Laboratory Standards Institute (CLSI) guidelines (49); the respective MICs were 0.25 mg/liter and 0.5 mg/liter for ciprofloxacin and 2 mg/liter and 4 mg/liter for meropenem. CLSI guidelines were used to define susceptibility and resistance criteria, with MICs of $\leq$0.5 mg/liter and $\geq$2 mg/liter for ciprofloxacin and $\leq$2 mg/liter and $\geq$8 mg/liter for meropenem (49). Thus, PAOΔ*mutS* was susceptible to both antibiotics, whereas CW44 was susceptible to ciprofloxacin and intermediate to meropenem. The mechanisms underlying increased meropenem MICs have been previously studied and included the inactivation of the porin OprD (insertion of 1 nucleotide [nt] at position 1200), the modification of the β-lactam target PBP3 (F533L mutation, known to be involved in β-lactam resistance), and the overexpression of the β-lactamase AmpC (deletion of nt 104 in *mpl*, a

negative regulator of *ampC*) (19). Hypermutability was defined as at least a 20-fold increase in the mutant frequency on rifampin-containing agar compared to that obtained for the control strain PAO1; the mutant frequencies of PAOΔ*mutS* and CW44 were 1,052-fold and 123-fold higher than that of PAO1, respectively (7, 19). The crystal violet assay (50) was carried out to confirm the biofilm formation capacity of both isolates.

***In vitro* dynamic biofilm model and antibiotic dosing schemes.** The CBR model (Bio Surface Technologies, Bozeman, MT, USA) was used to explore the microbiological response and emergence of resistance of planktonic and biofilm bacteria to the ciprofloxacin and meropenem treatments, in monotherapy and combinations, over 120 h, as described previously (51).

In brief, the CBR model consisted of three components connected in series: a 10-liter carboy containing sterile drug-free CAMHB–1% TSB, a 1-liter central glass reactor, and a carboy for waste collection. A peristaltic pump delivered the broth medium to the central reactor, where a magnetic stir bar operating at 130 rpm provided mixing. The system was maintained at 36°C. Biofilm formation occurred on removable polycarbonate coupons (diameter, 12.7 mm) located in eight polypropylene coupon holders suspended from the reactor lid (three coupons per holder); the total surface area of each coupon was 2.53 cm^2. Prior to each experiment, isolates were subcultured onto CAMHA and incubated at 36°C for 24 to 48 h (depending on the growth kinetics). Random colonies (2 to 3) were selected and grown overnight in 10 ml TSB, from which early-log-phase growth was obtained. At the commencement of the experiment, 1 ml of this early-log-phase bacterial suspension was inoculated into each reactor containing 350 ml TSB, and the flow of the system was halted for a 28-h conditioning phase to allow the bacteria to grow to form a biofilm. Conditioning involved the removal of all the broth from the reactor at 24 h to expel all planktonic bacteria. Subsequently, CAMHB–1%TSB was passed through the reactor for 4 h (flow rate of 11.67 ml/min) to ensure that planktonic bacteria present at the start of antibiotic treatment (i.e., 0 h) were those newly shed from the biofilm. The flow rate was changed to 1.39 ml/min at 0 h for all treatments, to represent a ciprofloxacin elimination half-life ($t_{1/2}$) of 2.9 h, reflecting patients with CF (52).

FDA-recommended daily doses of 1.2 g for ciprofloxacin and 6 g for meropenem were selected for administration. Ciprofloxacin was delivered as a 60-min i.v. infusion every 8 h via syringe drivers. The meropenem regimen was initiated with an appropriate loading dose at 0 h directly into the reactor, and thereafter, it was delivered as a continuous infusion (CI) by spiking the meropenem stock solution into the medium bottle so that all the media flowing through the system contained a constant concentration (unbound average steady-state concentration [fC_{ss}]) (Table 1) of meropenem. The medium bottles were stored in the fridge and changed every 24 h to avoid thermal degradation. Antibiotic concentrations in ELF are considered to be more relevant than those in plasma for pulmonary infections such as acute exacerbations in patients with CF. Thus, the ELF concentration-time profiles simulated in the CBR were determined based on population PK models from clinical studies in patients with CF and the ELF/plasma penetration ratios of ciprofloxacin and meropenem (52–57). The expected antibiotic plasma concentration-time profiles were simulated *in silico* using Berkeley Madonna (version 8.3.18) (58). The extents of penetration of ciprofloxacin (80%) (59, 60) and meropenem (30% and 60%) (36–39) into ELF were derived from multiple published studies using different groups of patients. Growth controls for both isolates were also included. Syringe drivers and pumps were tested and calibrated prior to the experiment, and the flow rate through the CBR was monitored during the experiment to ensure the optimal function of the system.

Quantification of bacterial killing and emergence of resistance. For viable-cell counting, 1-ml broth samples were collected from the reactor vessel at 0, 1, 2, 3, 5, 7, 24, 28, 48, 72, 96, and 120 h for planktonic bacteria. For biofilm bacteria, coupons were aseptically removed at 0, 3, 7, 24, 48, 72, 96, and 120 h and replaced with a blank holder. The coupons were carefully detached from the holders, washed twice in 10 ml of phosphate-buffered saline (PBS) (pH 7.4) to remove planktonic cells, and then stored in tubes containing 10 ml sterile PBS. Biofilm bacteria were extracted by three alternating 1-min cycles of vortex mixing and sonication at 43 kHz, followed by a final 1-min vortexing step (51). The total bacterial population was enumerated by manual plating of 100 μl of an appropriately diluted bacterial suspension onto drug-free CAMHA, followed by incubation for 24 h for PAOΔ*mutS* and for 48 h due to the slow growth of the hypermutable CW44 isolate at 36°C. Planktonic bacteria were expressed as $\log_{10}$ CFU per milliliter, and the number of bacteria recovered from coupons was expressed as $\log_{10}$ CFU per square centimeter. Less-susceptible subpopulations were quantified for planktonic and biofilm bacteria at 0 h (pretreatment) and 24, 72, and 120 h following the start of treatment, and 200 μl of an appropriately diluted sample was plated onto CAMHA supplemented with meropenem at 5 mg/liter or 10 mg/liter or with ciprofloxacin at 0.57 mg/liter or 1.25 mg/liter. The plates were incubated for 48 h. MICs were determined at 0 and 120 h by the agar dilution method for colonies isolated from antibiotic-containing plates to verify phenotypically the presence of stable resistance.

PK validation. Samples (1 ml) were serially collected from the growth control and treatment arms at multiple time points and immediately stored at −80°C. Meropenem and ciprofloxacin concentrations in CAMHB–1% TSB were measured using validated ultrahigh-performance liquid chromatography photodiode array (UHPLC-PDA) assays on a Shimadzu (Kyoto, Japan) Nexera UHPLC system coupled to a Shimadzu photodiode array detector. Test samples were assayed in a run order alongside matrix-matched calibrators (standards) and quality controls (QCs). A 5-μl aliquot of the sample was injected onto the UHPLC-PDA. For meropenem, an Onyx monolithic C_{18}, 50- by 2.0-mm analytical column (Phenomenex, Torrance, CA, USA) was used at room temperature. The binary mobile phase consisted of 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B). The gradient was programmed as 100% mobile phase A for 2.0 min that changed over 1.5 min to a mobile phase A/B ratio

of 10:90, which was held for 0.5 min, followed by reequilibration to 100% mobile phase A over 1.0 min. The mobile phase flow rate was 1.00 ml/min. For ciprofloxacin, a Shim-pack XR-ODS III, 2.0- by 50-mm (1.6-μm) analytical column (Shimadzu, Kyoto, Japan) was used, preceded by a Security Guard Ultra C_{18} guard cartridge (Phenomenex, Torrance, CA, USA) held at 30°C. The mobile phase was 87% phosphate buffer (0.1 M; pH 7) with 13% methanol and was delivered isocratically at 0.25 ml/min. The PDA monitored the UV spectrum from 250 to 340 nm. Approximate retention times and wavelengths for quantitation for each analyte peak were 2.7 min at 310 nm for meropenem and 8 min at 330 nm for ciprofloxacin. Samples were quantified against a calibration curve generated from the batch calibrators, and assay performance was ensured by batch acceptance criteria (61). Precisions were 1.4, 0.4, and 0.2% and accuracies were −0.1, −1.3, and −1.8% at 1.6, 16, and 160 mg/liter of meropenem in CAMHB–1% TSB, respectively. Precisions were 5.8, 1.3, 0.5, and 0.4% and accuracies were 3.6, −1.1, 3.5, and 5.4% at 0.2 mg/liter (lower limit of quantification [LLOQ]), 1.6 mg/liter, 16 mg/liter, and 160 mg/liter of ciprofloxacin in CAMHB–1% TSB, respectively.

Data analysis to describe bacterial killing and emergence of resistance. Synergy was defined as ≥2-$\log_{10}$ CFU/ml or CFU/cm² killing for the combination relative to the most active corresponding monotherapy at a specified time and ≥2 $\log_{10}$ CFU/ml below the initial inoculum (62). Combination regimens achieving enhanced killing with a reduction of ≥1 to <2 $\log_{10}$ CFU/ml or CFU/cm² compared to the most active corresponding monotherapy were also noted, as such an increase in killing may be important clinically. The log change in viable-cell counts was calculated as the difference of $\log_{10}$ CFU at each sample collection time during treatment from the $\log_{10}$ CFU at time zero. Mutant frequencies were calculated as the difference between the $\log_{10}$ CFU per milliliter ($\log_{10}$ CFU per square centimeter) on antibiotic-containing agar and the $\log_{10}$ CFU per milliliter ($\log_{10}$ CFU per square centimeter) on antibiotic-free agar at the same time point. Emergence of resistance was evaluated by comparing the bacterial counts on antibiotic-containing plates for the different treatments to those observed for the growth control.

Data availability. The figures and tables include the data from the reported studies.

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Chapter 5

Synergistic ceftazidime and tobramycin combinations for clinical hypermutable *Pseudomonas aeruginosa* isolates; an innovative dosing approach to enhance bacterial killing and mitigate resistance in a dynamic biofilm model

Preamble

The cephalosporin ceftazidime and the aminoglycoside tobramycin are considered first-line treatments for acute infective exacerbations caused by *P. aeruginosa* in CF, but their effects against planktonic and biofilm bacteria of hypermutable strains have not been investigated in a dynamic *in vitro* infection model. In addressing **Aim 4**, we systemically investigated the effect of lung fluid concentration-time profiles of tobramycin and ceftazidime in monotherapy and combination, on bacterial killing and resistance emergence of hypermutable *P. aeruginosa* clinical isolates, in the dynamic *in vitro* CBR over 120 h. Tobramycin was administered as either once-daily short-term intravenous infusions or by twice-daily inhalations and ceftazidime as a continuous infusion. This is the first study examining the pharmacodynamic effects of target site concentrations (i.e. lung fluid) for tobramycin administered by inhalation and ceftazidime against both modes of bacterial growth (planktonic and biofilm) in a dynamic model. The findings from this Chapter supported **Hypothesis 4** that continuous infusion of ceftazidime in combination with inhaled tobramycin provides more pronounced bacterial killing and suppression of resistant mutants compared to combinations containing tobramycin administered intravenously, against both planktonic and biofilm-embedded hypermutable *P. aeruginosa* bacteria. The results are presented similar to accepted Chapters in the section immediately following. The manuscript will be submitted soon to a peer-reviewed journal for publication.

Manuscript

Synergistic ceftazidime and tobramycin combinations for clinical hypermutable *Pseudomonas aeruginosa* isolates; an innovative dosing approach to enhance bacterial killing and mitigate resistance in a dynamic biofilm model

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Running title: Ceftazidime plus tobramycin against biofilm bacteria

Key words: Cephalosporins; aminoglycoside; inhalational tobramycin; biofilm; hypermutation; antibiotic resistance; pharmacokinetics/ pharmacodynamics; combination therapy; lung fluid; epithelial lining fluid; CBR

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Abstract

Pseudomonas aeruginosa chronically infects patients with cystic fibrosis and is associated with increased morbidity and mortality. Ceftazidime and tobramycin are considered first-line treatments. However, hypermutability and biofilm formation results in treatment failure due to selection of resistant mutants. We systematically investigated the pharmacodynamic effects of intravenous *versus* inhalation dosage regimens of tobramycin with and without intravenous ceftazidime in the dynamic *in vitro* CDC biofilm reactor (CBR). Two clinical hypermutable *P. aeruginosa* isolates CW30 (MIC_{CAZ} 0.5 mg/L, MIC_{TOB} 2 mg/L) and CW8 (MIC_{CAZ} 2 mg/L, MIC_{TOB} 8 mg/L) were investigated for 120 h. Clinically relevant treatments were: continuous infusion ceftazidime 9 g/day (33% lung penetration); intravenous tobramycin 10mg/kg Q24h (50% lung penetration); and tobramycin 300 mg Q12h as inhalation, and their combinations. Total and less-susceptible planktonic and biofilm bacterial counts were carried out over 120 h. All monotherapies were ineffective for both isolates, with a regrowth of planktonic (≥4.7log_{10} CFU/mL) and biofilm (>6.6log_{10} CFU/cm^2) bacteria, and amplification of less-susceptible planktonic and biofilm bacteria by 120 h. Both combination treatments demonstrated synergistic bacterial killing, not only for planktonic but also biofilm bacteria; however, greatest bacterial killing against both modes of bacterial growth was observed with the combination simulating tobramycin inhalation. In addition, the combination regimens resulted in a very substantial suppression of resistance of planktonic and biofilm bacteria to each of the antibiotics for both isolates. Thus, ceftazidime combinations with intravenous or, especially, inhaled tobramycin hold promise to treat challenging infections caused by hypermutable *P. aeruginosa* strains and warrant clinical investigation.

Introduction

Respiratory infections caused by Gram-negative pathogens, such as *Pseudomonas aeruginosa* in patients with cystic fibrosis (CF) are posing a significant challenge.[1] In CF, infective exacerbations with this pathogen and progressive pulmonary insufficiency are responsible for high morbidity and mortality.[2] Increased mucus viscosity and impaired mucociliary clearance facilitate the acquisition of early *P. aeruginosa* infection.[3] Over time, extensive use of antibiotics and significant pulmonary environmental pressures promote evolution and phenotypic shift of *P. aeruginosa*.[4] The hypermutator phenotype (with up to 1000-fold increased mutation rate), which results from defects in DNA repair or error avoidance systems, occurs in up to 65% of *P. aeruginosa* isolates from patients with CF.[5, 6] These strains are associated with the transition of early (planktonic phase) infection to a respiratory infection involving biofilm formation.[7, 8] The biofilm harbours phenotypic diversity and its matrix is dominated by alginate and exopolysaccharides that restrict the access of antibiotics to the infecting pathogen and also diminish their antibacterial activity due to low metabolic activity, less formation of reactive oxygen species (ROS), and adaptive stringent and stress-responses.[9, 10] Hypermutable *P. aeruginosa* strains develop resistance to antibiotics much more rapidly than non-hypermutable strains and are linked to increased multidrug-resistance.[11] Hypermutation in association with biofilm formation is widely recognised as a major problem for the effective treatment of *P. aeruginosa* respiratory infections in CF.[12-14]

The β-lactam antibiotic ceftazidime and the aminoglycoside tobramycin are commonly used intravenously as first-line treatments for *P. aeruginosa* exacerbations. Their use as monotherapy is not recommended and information about rational dosing of this antibiotic combination in CF is limited.[15, 16] *P. aeruginosa* has a large armamentarium of resistance mechanisms and can rapidly develop resistance following suboptimal antibiotic exposures, increasing the risk of therapeutic failure.[17] In addition, for most antibiotics (including ceftazidime and tobramycin) the minimal biofilm inhibitory concentration is usually more than

one two-fold dilution higher than the MIC for planktonic bacteria, further complicating the treatment of acute infective exacerbations in patients with CF.[18]

While inhaled tobramycin is widely used in attempts to eradicate early acquisition of *P. aeruginosa* and control chronic infections, it is not usually employed in the management of acute infective exacerbations of hypermutable *P. aeruginosa*.[19-21] This mode of administration ensures delivery of high concentrations of the antibiotic into lung fluid.[22-25] The effect of the tobramycin concentrations that can be achieved with inhalation against hypermutable *P. aeruginosa* embedded in biofilms and in planktonic form has never been explored in a dynamic biofilm model, such as the Center for Disease Control biofilm reactor (CBR). The main objective of the present study was to systematically investigate the effect of clinically relevant lung fluid concentration-time profiles of ceftazidime and tobramycin (intravenous *versus* inhalation), as monotherapy and in combination, against clinical isolates of hypermutable *P. aeruginosa* in the dynamic *in vitro* CBR model. The time-course of bacterial killing and resistance emergence or suppression of both planktonic and biofilm bacteria was examined over 120 h in the CBR.

Materials and Methods

Bacterial isolates: Two clinical hypermutable *P. aeruginosa* isolates (CW30 and CW8) were examined. The clinical isolates were obtained from the sputum of patients with CF and respiratory infection at the Alfred Hospital (Melbourne, Australia) and had inactivating mutations within *mutL*.[26] Hypermutability was defined as at least a 20-fold increase in mutant frequency on rifampicin-containing agar in comparison to that obtained for the reference strain PAO1; the mutant frequencies of CW30 and CW8 were 84.9-fold and 141.0-fold higher than that of PAO1, respectively.[5, 26] The biofilm formation capacity of both isolates was tested by the crystal violet assay.[27]

Antibiotics: Stock solutions of ceftazidime (Waterstone Technology, Carmel, IN, USA; Lot WS16174) and tobramycin (AK Scientific, Union City, MD, USA; Lot LC24138) were prepared

in sterile Milli-Q® water and filter sterilized with a Millex-GV 0.22 µm polyvinylidene difluoride (PVDF) syringe filter (Merck Millipore Ltd., Cork, Ireland) prior to each experiment.

Susceptibility testing: The MICs of ceftazidime and tobramycin were determined for each isolate in triplicate on each of three separate days by using agar dilution as per Clinical and Laboratory Standards Institute (CLSI) guidelines;[28] the MICs were 0.5 mg/L and 2 mg/L for ceftazidime, and 2 mg/L and 8 mg/L for tobramycin for CW30 and CW8, respectively. Susceptibility and resistance were interpreted as a MIC ≤8 mg/L and ≥32 mg/L for ceftazidime, and ≤4 mg/L and ≥16 mg/L for tobramycin, as per CLSI guidelines.[28] Thus, CW30 was susceptible to both antibiotics, whereas CW8 was susceptible to ceftazidime and intermediate to tobramycin. In addition, CW8 was identified previously as carbapenem-resistant and multidrug-resistant (MDR) based on agar dilution MICs,[29] where MDR was defined as non-susceptibility to at least one antimicrobial agent in three or more antimicrobial categories.[30] We have previously reported the following mutations in genes associated with resistance to β-lactams and aminoglycosides, for isolate CW8 - *ampR* and *mpl* (associated with increased expression of AmpC), *mexF* (MexEF-OprN efflux pump) and *oprD* (outer membrane protein); and for CW30 - *mexF* (MexEF-OprN efflux pump) and *mexZ* (MexXY-OprM efflux pump).[26] It is important to note that genomic data do not always correlate with susceptibility findings.[31-33]

Media: All CBR experiments used cation-adjusted Mueller-Hinton broth containing 25 mg/L Ca^{2+} and 12.5 mg/L Mg^{2+} (CAMHB) and was supplemented with 1% tryptic soy broth (TSB; BD, Sparks, MD, USA). For viable-cell counting cation-adjusted Mueller-Hinton agar (CAMHA; BD, Sparks, MD, USA) was used and drug-containing agar plates were prepared on the day of experiment by adding appropriate volumes of antibiotic stock solutions to CAMHA.

Antibiotic dosing schemes: Doses of 9 g/day for intravenous ceftazidime were selected for simulation in the CBR following EMA recommendations, whereas 10 mg/kg/day for intravenous tobramycin and 300 mg 12-hourly for inhalation of tobramycin were selected following FDA-recommendations. To represent antibiotic concentrations in acute exacerbations in patients with CF with pulmonary infections, lung fluid pharmacokinetics were chosen to be more

clinically relevant than those in plasma. Thus, the plasma concentration-time profiles of ceftazidime and tobramycin following intravenous dosing were derived from population pharmacokinetic models from clinical studies in patients with CF.[34, 35] For the regimens simulating intravenous administration in the CBR, the extents of lung epithelial lining fluid (ELF) penetration of ceftazidime (33%) and tobramycin (50%) were derived from published studies.[36-41] The slightly longer elimination half-life of tobramycin from ELF compared to plasma was accounted for.[36, 37] The lung fluid concentration-time profiles of tobramycin following inhaled administration were informed by clinical studies that quantified tobramycin in ELF[24, 42] or induced and expectorated sputum[25] at different time points following treatment with tobramycin solution for inhalation. The expected antibiotic concentration-time profiles in lung fluid were simulated *in silico* using Berkeley Madonna (version 8.3.18).[43] These profiles were subsequently simulated in the CBR model.

CBR model and antibiotic administration: Experiments to investigate the antibacterial activity and emergence or suppression of resistance by the dosing schemes of ceftazidime and tobramycin alone and in combinations were conducted over 120 h using a dynamic CBR model (Bio Surface Technologies, Bozeman, MT, USA), as described previously[29] and briefly below. Prior to each experiment, isolates were subcultured onto CAMHA and incubated at 36˚C for 48 h. Colonies (2-3) were randomly selected and grown overnight in 10 mL of TSB, from which early-log-phase growth was obtained.

At the commencement of the experiment, 1 mL of the early-log-phase bacterial suspension was inoculated into each reactor containing 350 mL TSB and the flow of the system was turned off for a 24 h period, to allow the bacteria to replicate and form biofilm. The system was maintained at 36˚C. Biofilm formation occurred on removable polycarbonate coupons located in eight polypropylene coupon holders suspended from the reactor lid (three coupons per holder); with a total surface area of 2.53 cm^2/coupon. After 24 h all the broth was removed to expel all planktonic bacteria from the reactor and CAMHB with 1% TSB was passed through the reactor for 4 h (flow rate 11.67 mL/min) to ensure planktonic bacteria present at the start

of antibiotic treatment (*i.e.* 0 h) were newly shed from the biofilm. Broth medium was delivered *via* a peristaltic pump to the central reactor where a magnetic stir bar (130 rpm) provided mixing. After a 4-h conditioning phase the flow rate of the system was adjusted to 1.16 mL/min at 0 h, before starting treatment, to represent a tobramycin elimination half-life ($t_{1/2}$) in lung fluid of 3.5 h derived from published studies in patients with CF.[34, 36, 44] Growth controls for both isolates were also included and some monotherapy and combination treatments were tested in two replicates. Syringe drivers and pumps were tested and calibrated prior to the experiment and the flow rate through the CBR was monitored during the experiment to ensure the optimal function of the system.

Ceftazidime dosing was started with a loading dose at 0 h directly into the reactor, followed by delivery as continuous infusion (CI) achieved by spiking an appropriate volume of ceftazidime stock solution into the feeder reservoir so that all the sterile media flowing through the system contained a constant concentration (steady-state concentration, fC_{ss}; Table 1) of ceftazidime. To avoid thermal degradation the feeder reservoir was kept in the fridge and changed every 24 h. Tobramycin was delivered *via* syringe driver over 30 min every 24 h and over 15 min every 12 h to simulate intravenous and inhalational dosing, respectively. As tobramycin does not accumulate following multiple intravenous administration, no loading dose was required to achieve steady-state concentrations; however, for tobramycin inhalation a small loading dose equivalent to the amount needed to attain fC_{min} (Table 1) was administered along with the first maintenance dose.

Sampling and quantification of microbiological response: For planktonic bacteria viable counting, broth samples (1 mL) were serially collected at 0, 1, 2, 3, 5, 7, 24, 28, 48, 72, 96 and 120 h from each treatment reactor. For biofilm bacteria, coupon holders were aseptically removed from each treatment reactor at 0, 3, 7, 24, 48, 72, 96 and 120 h and replaced with a sterile blank holder. The coupons were carefully processed by removing them from the holders followed by washing twice with 10 mL of phosphate-buffered saline (PBS, pH 7.4) to remove planktonic cells. All 3 coupons recovered at each time point from every arm were stored in

tubes containing 30 mL of sterile PBS. Later, biofilm bacteria were extracted by alternating cycles of vortex, mixing and sonication at 43 kHz for 10 mins.[29] The quantification of total bacterial populations for planktonic and biofilm bacteria was carried out by plating of 100 µL of appropriately diluted bacterial suspension manually onto drug-free CAMHA followed by incubation for 48 h at 36°C. Less susceptible subpopulations were quantified at baseline (just prior to commencing treatment), and during the treatment at 24, 72 and 120 h by plating 200 µL of appropriately diluted sample onto CAMHA supplemented with ceftazidime at 2.5 or 6 mg/L and 10 mg/L, or tobramycin at 5 or 10 mg/L and 10 or 20 mg/L for CW30 and CW8, respectively. The plates were incubated for 48 h. Planktonic bacteria were expressed as $\log_{10}$ CFU/mL and biofilm bacteria recovered from coupons were expressed as $\log_{10}$ CFU/cm^2.

PD analysis: Microbiological responses to monotherapy and combination therapy were examined using the log-change method by calculating the change in $\log_{10}$ CFU/mL (planktonic bacteria) or $\log_{10}$ CFU/cm^2 (biofilm bacteria) from time 0 h (CFU_0) to the time of each sample during treatment (CFU_t). Synergy was considered as ≥2 $\log_{10}$ CFU/mL or $\log_{10}$ CFU/cm^2 more killing for the combination relative to the most active corresponding monotherapy at a specified time and, for planktonic cells, a viable cell count ≥2 $\log_{10}$ CFU/mL below the initial inoculum.[45] Combination regimens achieving a reduction of ≥1 to <2 $\log_{10}$ CFU/mL or $\log_{10}$ CFU/cm^2 compared to the most active corresponding monotherapy at the same time were considered to represent an enhanced bacterial killing, as this may be clinically important. Mutant frequencies were calculated at various time points as the difference of $\log_{10}$ CFU/mL or $\log_{10}$ CFU/cm^2 on antibiotic-containing agar to antibiotic-free agar at the same time point. Emergence or suppression of resistance was evaluated at 120 h by comparing the bacterial counts on antibiotic-containing plates for the different treatments with those observed for the growth control.

Results

Microbiological response

Viable-cell count profiles for total populations of planktonic and biofilm bacteria (each observation was based on 3 coupons) are shown in Figure 1 for both isolates, while the corresponding profiles for less-susceptible populations of CW30 and CW8 are in Figures 2 and 3, respectively. Log changes in viable-cell counts of total bacteria and mutant frequencies are shown in Tables 2 and 3, respectively.

Planktonic bacteria: The initial inocula (mean ± SD) in all arms were 7.51 ± 0.13 $\log_{10}$ CFU/mL (n=8) for CW30 and 7.26 ± 0.32 $\log_{10}$ CFU/mL (n=7) for CW8. The total population of CW30 grew rapidly in control arms and plateaued by 24 h at ~8.8 $\log_{10}$ CFU/mL (Figure 1A). By 120 h, less-susceptible populations grew to ~3.7 and 1.7 $\log_{10}$ CFU/mL on agar containing 2.5 mg/L and 10 mg/L of ceftazidime, and to ~4.3 and 3.4 $\log_{10}$ CFU/mL on agar containing 5 mg/L and 10 mg/L of tobramycin, respectively (Figure 2A, C, E, G). The total population of CW8 grew to ~8.4 $\log_{10}$ CFU/mL by 24 h and plateaued at ~8.7 $\log_{10}$ CFU/mL by 48 h (Figure 1C). Less-susceptible populations grew on agar containing 6 and 10 mg/L of ceftazidime and 10 and 20 mg/L of tobramycin approximately in proportion to the growth of the total bacterial population (Figure 3A, C, E, G and Table 3).

For CW30, ceftazidime monotherapy produced an initial killing of ~2.9 $\log_{10}$ CFU/mL at 7 h followed by slow regrowth to ~7 $\log_{10}$ CFU/mL by 120 h, which was ~2 $\log_{10}$ CFU/mL below the growth control (Figure 1A and Table 2). Amplification of ceftazidime less-susceptible populations was observed at 120 h, such that the majority of bacteria in the total population (~6.8 $\log_{10}$ CFU/mL) grew on plates containing 2.5 mg/L and 10 mg/L of ceftazidime (Figure 2A, C and Table 3). Amplification of resistance was observed at 120 h; on agar plates containing 2.5 mg/L and 10 mg/L ceftazidime there was ~2.9 log and ~4.9 log increase, respectively, of the ceftazidime-resistant population as compared to control values. With CW8, ceftazidime monotherapy achieved an initial killing of ~2.3 $\log_{10}$ CFU/mL at 7 h followed by regrowth close to the control values by 72 h (Figure 1C and Table 2). Less-susceptible

populations were observed at 120 h, such that a large proportion of the entire population grew on plates containing 6 or 10 mg/L of ceftazidime (Figure 3A, C and Table 3). Amplification of resistance was observed with a ~2.7 log and ~4 log increase of ceftazidime-resistant bacteria on these respective drug-containing plates, in comparison to growth control.

For CW30, tobramycin monotherapy simulating intravenous dosing produced an initial killing of ~2 $\log_{10}$ CFU/mL at 7 h followed by steady regrowth to ~7.6 $\log_{10}$ CFU/mL by 48 h, with further regrowth close to the growth control at 120 h (Figure 1A and Table 2). With this treatment, less-susceptible populations increased rapidly, with ~7.9 $\log_{10}$ CFU/mL growing on plates containing 5 mg/L of tobramycin and ~7.2 $\log_{10}$ CFU/mL on plates containing 10 mg/L. Amplification of resistance was evident with an increase of ~3.8 log of the tobramycin-resistant population at 120 h in comparison to growth control (Figure 2E, G and Table 3). With tobramycin monotherapy simulating inhalation dosing an extensive initial killing of ~4.2 $\log_{10}$ CFU/mL was achieved at 7 h with regrowth remaining suppressed at ~4.7 $\log_{10}$ CFU/mL at 120 h which was ~4 log below the viable count with intravenous tobramycin (Figure 1A and Table 2). Less-susceptible populations of ~3.8 $\log_{10}$ CFU/mL on plates containing 5 mg/L of tobramycin and ~2.3 $\log_{10}$ CFU/mL on plates containing 10 mg/L of tobramycin were obtained at 120 h, which were 4-5 log below the less-susceptible populations observed with intravenous tobramycin. With CW8, tobramycin monotherapy simulating intravenous dosing produced an initial killing of ~2.3 $\log_{10}$ CFU/mL at 7 h followed by steady regrowth close to growth control by 48 h. With tobramycin inhalation dosing, ~2.7 $\log_{10}$ CFU/mL of bacterial killing was achieved at 7 h with a further ~0.9 log increase in killing at 28 h. Subsequently, there was slow regrowth to a viable count of ~5.9 $\log_{10}$ CFU/mL at 120 h which was ~2.6 log below the count with the treatment simulating intravenous administration (Figure 1C and Table 2). Less-susceptible populations increased approximately in proportion to the growth of the total bacterial population. At 120 h the less-susceptible populations exceeded the values observed for the growth control, but were lower than those of the intravenous treatment (Figure 3E, G and Table 3).

For CW30, the combination simulating intravenous tobramycin produced rapid initial killing of ~2.7 $\log_{10}$ CFU/mL at 3 h which increased to killing of ~3.2 $\log_{10}$ CFU/mL at 7 h and ~3.5 $\log_{10}$ CFU/mL killing by 28 h (Figure 1A). Enhanced bacterial killing (reduction of ≥1 to <2 $\log_{10}$ CFU/mL compared to the most active corresponding monotherapy) was observed at 72 h, and synergy (≥2 $\log_{10}$ CFU/mL compared to the most active monotherapy) at 96 and 120 h (Table 2). The combination simulating tobramycin inhalation achieved extensive initial killing of ~3.9 $\log_{10}$ CFU/mL at 3 h with killing of ~5.4 and ~7.5 $\log_{10}$ CFU/mL at 7 and 28 h, respectively. Across the remainder of the treatment period to 120 h, viable counts were close to or below the limit of counting (Figure 1A). Enhanced bacterial killing was observed as early as 1 and 5 h, and synergy was evident at 28, 96 and 120 h (Table 2). At 120 h with both of these combination regimens it was noticeable that no colonies were present on agar plates containing 2.5 or 10 mg/L of ceftazidime and 5 or 10 mg/L of tobramycin (Figure 2A, C, E, G). Thus, very extensive suppression of resistance was observed.

For CW8, with the combination simulating intravenous tobramycin bacterial killing of ~2.8 $\log_{10}$ CFU/mL was achieved at 3 h with killing increasing to ~3.2 and ~3.8 $\log_{10}$ CFU/mL at 7 and 28 h, respectively (Figure 1C and Table 2). Thereafter, the viable cell count remained at ~4 log CFU/mL across the period to 120 h. Enhanced bacterial killing was achieved with this combination at 5 and 28 h, and synergistic killing at 48 h and beyond (Table 2). Growth of less-susceptible bacteria of ≤3.2 $\log_{10}$ CFU/mL was obtained on agar containing 6 or 10 mg/L ceftazidime, counts that were ~1 to 2.5 $\log_{10}$ CFU/mL below the growth control values (Figure 3A, C). No colonies were detected at 120 h on agar containing 10 or 20 mg/L of tobramycin (Figure 3E, G). With the combination simulating inhalation tobramycin, ~3.5 $\log_{10}$ CFU/mL of bacterial killing was achieved at 3 h with killing of ~3.7 and ~5.2 $\log_{10}$ CFU/mL at 7 and 28 h, respectively. Viable counts plateaued at ~3.7 $\log_{10}$ CFU/mL from 72 to 120 h (Figure 1C). This combination produced enhanced bacterial killing at 1 and 5 h and across all samples from 24 to 96 h inclusive, with synergy observed at 120 h (Table 2). Less-susceptible bacteria of ~1.2 $\log_{10}$ CFU/mL at 120 h were retrieved from plates containing 6 or 10 mg/L of ceftazidime,

counts that were ~3 to 4 $\log_{10}$ CFU/mL below the corresponding growth control counts and ~1.5 log below the counts for the combination with intravenous tobramycin. With the inhalation combination, no colonies were detected on tobramycin-containing drug plates (Figure 3A, C, E, G and Table 3).

Biofilm-embedded bacteria: The initial inocula (mean ± SD) in all arms were 8.30 ± 0.03 $\log_{10}$ CFU/cm^2 (n=3 coupons x8) and 7.58 ± 0.06 $\log_{10}$ CFU/cm^2 (n=3x7) for CW30 and CW8, respectively. By 24 h the growth controls of both isolates grew to ~8.9 $\log_{10}$ CFU/cm^2 and ~8.4 $\log_{10}$ CFU/cm^2, respectively, and plateaued until 120 h (Figure 1B and D). Colonies on drug-containing agar increased approximately in proportion to growth of the total bacterial population (Figures 2 and 3B, D, F, H).

For CW30, treatments simulating ceftazidime and intravenous tobramycin monotherapy produced ≤0.7 $\log_{10}$ CFU/cm^2 killing at 7 h followed by regrowth close to the growth control. With tobramycin inhalation monotherapy ~2.6 $\log_{10}$ CFU/cm^2 bacterial killing was achieved at 48 h and maximal killing of ~3.6 $\log_{10}$ CFU/cm^2 was observed at 72 to 96 h. Across the period from 24 to 120 h, viable counts were ~2.1 to 4.4 log below the corresponding counts for intravenous tobramycin monotherapy (Figure 1B and Table 2). With ceftazidime and intravenous tobramycin monotherapy, substantial increases in less-susceptible populations occurred and amplification of resistance was observed at 120 h (Figure 2B, D, F, H and Table 3). For the treatment simulating tobramycin inhalation, at 120 h ~5.1 and 4.7 $\log_{10}$ CFU/cm^2 was observed on agar containing 5 or 10 mg/L of tobramycin which were ~0.7 and ~3.7 log below the respective counts for the growth control and the intravenous tobramycin treatment. The combination simulating intravenous tobramycin produced enhanced bacterial killing at 24 and 48 h, and synergy from 72 to 120 h; across the period from 48 to 120 h bacterial killing of ~3.2 to 4.1 $\log_{10}$ CFU/cm^2 was observed (Figure 1B and Table 2). The combination with tobramycin inhalation produced extensive killing and only a negligible number (~1 $\log_{10}$ CFU/cm^2) of colonies was retrieved at 120 h; the viable count at this time was almost 3 $\log_{10}$ CFU/cm^2 lower than that observed with the intravenous tobramycin combination. The

tobramycin inhalation combination regimen produced enhanced or synergistic killing from 48 to 120 h inclusive (Figure 1B and Table 2). After 120 h of treatment with combinations, suppression of resistance was observed. No colonies were present on agar containing 5 or 10 mg/L of ceftazidime or 10 mg/L of tobramycin for both combinations. However, a count of ~2.9 $\log_{10}$ CFU/cm^2 was observed on plates containing 5 mg/L of tobramycin with the combination simulating intravenous tobramycin, a count that was ~3 log below the count for the growth control (Figure 2B, D, F, H).

For CW8, following 7 h of treatment the activity of all monotherapies produced <0.6 $\log_{10}$ CFU/cm^2 of bacterial killing. After that, regrowth occurred with the ceftazidime and intravenous tobramycin monotherapy treatments such that bacterial counts were <1 $\log_{10}$ CFU/cm^2 below those of the growth control across the 72 to 120 h window (Figure 1D and Table 2). Greater bacterial killing was observed with the tobramycin inhalation monotherapy treatment; maximum killing of ~1.2 $\log_{10}$ CFU/cm^2 was observed at 72 h and at 120 h the bacterial count was ~2 $\log_{10}$ CFU/cm^2 below that of the growth control. Substantial increases in less-susceptible populations for both ceftazidime (6 and 10 mg/L on agar) and tobramycin (10 and 20 mg/L on agar) were observed for all the monotherapies (Figure 3B, D, F, H and Table 3). Amplification of resistance was observed at 120 h with ~1.3 to ~4.3 $\log_{10}$ CFU/cm^2 increase of the ceftazidime- and tobramycin-resistant bacteria in comparison to corresponding control values. In comparison, both combinations produced substantially greater antibacterial activity. With the combination simulating intravenous tobramycin, enhanced activity was observed at 24 and 48 h, while synergistic bacterial killing was observed from 72 h onwards (Figure 1D and Table 2). For the combination simulating tobramycin inhalation, enhanced activity was observed at 24 h and synergy from 48 to 120 h. Across the latter period, the viable count remained below ~4.5 $\log_{10}$ CFU/cm^2 and the bacterial killing was ~1.3 to ~1.7 $\log_{10}$ CFU/cm^2 greater than occurred with the intravenous tobramycin combination. No colonies were observed on agar plates containing 6 or 10 mg/L of ceftazidime or 10 or 20 mg/L of tobramycin for both combinations (Figure 3B, D, F, H and Table 3).

Discussion

Recurrent acute infective exacerbations of chronic *P. aeruginosa* respiratory infections in patients with CF often require long, repeated and aggressive antimicrobial therapies to decrease the bacterial burden and restore the baseline lung function.[12, 46] Ceftazidime and tobramycin are considered first-line treatments for pulmonary exacerbations.[47, 48] However, the antibacterial effect of ceftazidime with tobramycin against *P. aeruginosa* isolates from patients with CF has never been explored in a dynamic biofilm model. This study systematically investigated bacterial killing and resistance suppression of intravenous *versus* inhalation dosage regimens of tobramycin with and without ceftazidime against two clinical hypermutable *P. aeruginosa* isolates in the CBR. The most important findings of the study were that simulated combination regimens of ceftazidime and tobramycin, with the latter administered either intravenously or by inhalation, were able to achieve synergy against not only planktonic but also biofilm bacteria, with the greatest bacterial killing occurring for the combination regimen simulating inhalation of tobramycin. In addition, the combination regimens resulted in a very substantial suppression of resistance of both bacterial growth forms to each of the antibiotics.

For β-lactams, including ceftazidime, traditionally the most predictive PK/PD index driving the antibacterial activity is the duration of the dosing interval over which the unbound concentration remains above a multiple of the MIC of the infecting pathogen ($fT_{>MIC}$).[49-53] In a large clinical trial in which ceftazidime was used to treat nosocomial pneumonia favourable microbiological and clinical outcomes were more likely when $fT_{>MIC}$ was >45%.[54] Since the maximum rate of bacterial killing occurs at an unbound ceftazidime concentration ~4× MIC, it was suggested that continuous infusion of ceftazidime to maintain unbound concentrations 4-fold higher than the MIC should maximise efficacy.[52, 55]

In the present CBR study, 9 g/day ceftazidime was simulated as a CI to maximize $fT_{>MIC}$. For ceftazidime monotherapy against both isolates the unbound concentrations remained well above the respective MIC at all times. For CW30 the ceftazidime concentration was 48× MIC across the entire study duration; the corresponding value for CW8 was 12× MIC. Even with

these high and sustained levels of exposure, ceftazidime monotherapy was unable to suppress the regrowth and resistance emergence for both planktonic and biofilm bacteria for both isolates. In contrast, *in vitro* studies conducted against clinical strains of *P. aeruginosa* with pre-existing resistant mutants suggested that an unbound ceftazidime concentration >3.8-fold higher than the MIC was needed to suppress the amplification of resistant subpopulations.[56] That study examined planktonic bacteria in a hollow-fibre infection model, whereas in the present study the interaction and cycling of biofilm and planktonic bacteria may have decreased the ability to suppress amplification of resistant subpopulations. In the current study, biofilm bacteria were less susceptible to ceftazidime than planktonic bacteria. Ceftazidime has a high binding affinity to PBP3 and PBP1a of *P. aeruginosa*.[57] Expression of PBP3 is downregulated at stationary phase,[58] a growth stage with physiological similarity to biofilm bacteria.[59] In addition, low metabolic activity of subpopulations located in the inner parts of the biofilm due to oxygen limitation contributes to tolerance of biofilm cells to the effects of ceftazidime.[60] In a recent CBR study on non-hypermutable *P. aeruginosa* clinical isolates, ceftazidime administered in a regimen simulating 2 g every 8 h was ineffective, which was not surprising given that the isolates were ceftazidime-resistant.[61] The failure of ceftazidime 9 g/day as CI in the current study strongly argues against the use of monotherapy against hypermutable and biofilm-forming *P. aeruginosa* strains, because such strains can readily develop resistance and become MDR due to increased mutation.[62]

For aminoglycosides such as tobramycin, antibacterial activity has been correlated with the ratio of unbound exposure across a 24 h period to MIC (*f*AUC/MIC) and the ratio of unbound maximum concentration to MIC (fC_{max}/MIC).[63] In serious bacterial infections, *f*AUC/MIC of >70 and an fC_{max}/MIC of 8-10 have been proposed as targets for clinical success.[64] In the present study, tobramycin was administered representing two modes of delivery; intravenous and inhalation. For the regimen simulating intravenous administration (10 mg/kg every 24 h), *f*AUC/MIC and fC_{max}/MIC were 32.2 and 6.1, respectively, for CW30; the corresponding values for CW8 were 8.1 and 1.5. The exposure values for inhalation delivery

(300 mg every 12 h) were 464 and 50 for *f*AUC/MIC and fC_{max}/MIC for CW30, and 116 and 12.5, respectively, for CW8. The *f*AUC/MIC and fC_{max}/MIC values achieved with the intravenous regimen did not reach the above-mentioned PK/PD targets for either isolate, but the targets were exceeded with inhalation delivery against both isolates. However, neither of the tobramycin regimens in monotherapy was able to maintain suppression of regrowth for both planktonic and biofilm bacteria, even though the tobramycin inhalation regimen comfortably achieved the target PK/PD values. This result agreed with our previous *in vitro* studies where tobramycin monotherapy failed to inhibit regrowth of planktonic hypermutable *P. aeruginosa* even with *f*AUC/MIC exposure values substantially higher than 70.[65, 66] For cationic antibiotics such as tobramycin the antibacterial activity against biofilm bacteria is decreased *via* chelation with extracellular DNA fragments in the biofilm matrix[10] and also due to over expression of MexXY-OprM which is particularly common in strains from patients with CF.[10] The results of the current study indicate that tobramycin monotherapy, even when administered by inhalation, is not an appropriate treatment option for the treatment of pulmonary infections caused by hypermutable *P. aeruginosa*.

The combination regimen containing intravenous tobramycin increased bacterial killing and decreased emergence of resistant subpopulations. The increased antibacterial effect culminated in enhanced or synergistic killing of planktonic and biofilm bacteria of both isolates across the last 3 to 4 days of the study period. The extent of the increased killing of biofilm bacteria, relative to either antibiotic administered as monotherapy, was especially marked. However, the largest antibacterial effect occurred with the combination containing tobramycin inhalation. Against both isolates, that combination regimen produced enhanced or synergistic bacterial killing of planktonic bacteria on each of the 5 days of the study, and of biofilm bacteria across the last 3 to 4 days over which time at least a 3 to 4 $\log_{10}$ reduction in bacterial count was observed relative to inhaled tobramycin alone. An additional key finding of both combination regimens was resistance suppression. The greater antibacterial activity observed with both combination regimens was notable, given the isolates were strong hypermutators

and at baseline had mutations in genes associated with resistance to β-lactams and aminoglycosides.[26] A previous study also investigated the ceftazidime and tobramycin combination in vitro in 24-h static concentration time-kill and dynamic two-compartment PK/PD model studies against a double resistant P. aeruginosa isolate from a patient with CF.[80] While synergy was observed, the study only examined planktonic bacteria over 24 h and did not mention whether the isolate was hypermutable.[80]

The enhanced and synergistic bacterial killing in the current study may be the result of the difference between the two antibiotics in mechanisms of action and resistance. Ceftazidime inhibits cell wall synthesis *via* binding to PBPs, and major mechanisms of resistance in *P. aeruginosa* clinical isolates involve chromosomally-mediated Amp*C* β-lactamase overexpression, enzymatic inactivation *via* β-lactamases and reduced affinity of PBPs.[17, 67] Tobramycin blocks protein synthesis, but also disrupts the outer bacterial membrane very likely resulting in increased ceftazidime concentrations in the periplasmic space where the PBPs are located.[68-70] Resistance mechanisms of *P. aeruginosa* against aminoglycosides include increased expression of MexXY-OprM, target-site modification, enzymatic cleavage and reduced outer membrane permeability.[71-75] In addition, for bacteria embedded in biofilm a number of other mechanisms can lead to tolerance against antibiotics, including reduced access of antimicrobials, oxygen limitation and low metabolic activity of biofilm bacteria.[10] The mechanism by which the combination regimens achieved such remarkable activity towards biofilm bacteria is unknown, but may be related to the ability of tobramycin to inhibit adhesion and microcolony formation during cycling of biofilm and planktonic cells.[76]

There are a number of potential advantages of administering antibiotics for treatment of lung infections by the inhalation route.[77] These advantages include the ability to achieve a very much higher lung-to-plasma concentration ratio than can be achieved with intravenous administration. This means that for antibiotics with narrow therapeutic windows (*e.g.* tobramycin) it is possible to achieve concentrations in lung much higher than can be safely achieved with intravenous administration; for tobramycin this implies reduced risk of

nephrotoxicity. For the last few decades antibiotics have been administered by inhalation for the management of chronic lung infections, including those occurring in patients with CF.[78, 79] A recent Cochrane systematic review considered the randomised controlled trials that have been conducted in people with CF with a pulmonary exacerbation in whom treatment withinhaled antibiotics was compared to placebo, standard treatment or another inhaled antibiotic for between one and four weeks.[19] Four trials with 167 participants were finally included in the review. Unfortunately, due to heterogeneity in trial design, high risk from lack of blinding, difficulty in assessing risk of bias, and lack of statistical power, the systematic review was unable to demonstrate whether or not one treatment was superior to the other. The authors concluded that further research is needed to establish whether inhaled tobramycin may be used as an alternative to intravenous tobramycin for pulmonary exacerbations.[19] The results of the study reported here lend strong support for the conduct of well-designed clinical trials to evaluate the delivery of tobramycin by inhalation in patients having pulmonary exacerbations.

The strengths of this study include the following: it is the first study to examine the activity of clinically relevant regimens of ceftazidime and tobramycin in monotherapy and in combination against isolates of *P. aeruginosa* that were hypermutable, representing a worst case scenario; the impact of delivering tobramycin by inhalation *versus* intermittent intravenous infusion was investigated; the effects on both planktonic and biofilm bacteria were examined in a dynamic model over 5 days; and, the time-courses of both total and resistant subpopulations in response to the monotherapy and combination regimens were characterized. The study also has limitations. Firstly, like other *in vitro* infection models, the dynamic biofilm model lacks an immune system and therefore the responses observed reflect the effects of the antibiotics only. Secondly, while our previous studies on the isolates provided data on mutations in resistance genes present prior to treatment,[26] we did not undertake genomic studies on emergent resistant populations of failed monotherapy regimens. Finally, it may also have been helpful to explore possible changes in biofilm structure in response to the different regimens.

In conclusion, the study has provided evidence that ceftazidime and tobramycin when administered in monotherapy against hypermutable P. aeruginosa are unable to provide sustained reduction in total and resistant subpopulations of both planktonic and biofilm bacteria. This supports guideline recommendations that antibiotics should not be used in monotherapy regimens for treatment of pulmonary exacerbations. Indeed, the study demonstrated the enhanced and synergistic activity of combination therapy, especially with tobramycin administered by inhalation. The latter finding reinforces calls for appropriate clinical studies to investigate the administration of antibiotics by inhalation in the treatment of pulmonary exacerbations.

Data availability: The figures and tables include the data from the reported studies.

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Figure 1. Total viable counts for growth control and treatments with ceftazidime (CAZ) and/or tobramycin (TOB) with clinically relevant lung fluid concentration-time profiles. Samples were from the media within the reactor, *i.e.* planktonic bacteria; and from coupons, *i.e.* biofilm bacteria. The y-axis starts at the limit of counting. The results from treatment arm C for CW30 and A + C for both isolates are presented as average ± SE of two replicates.

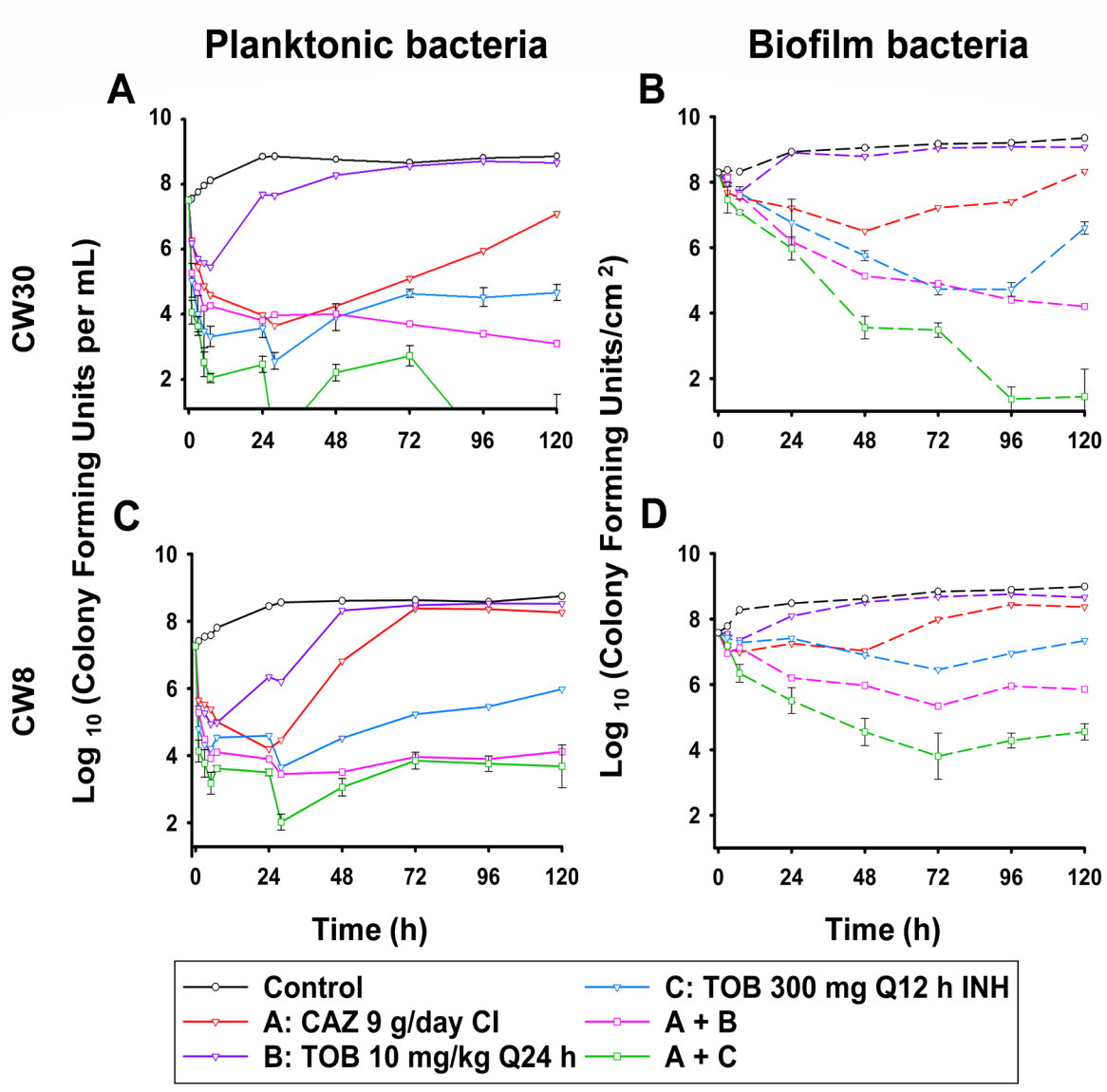
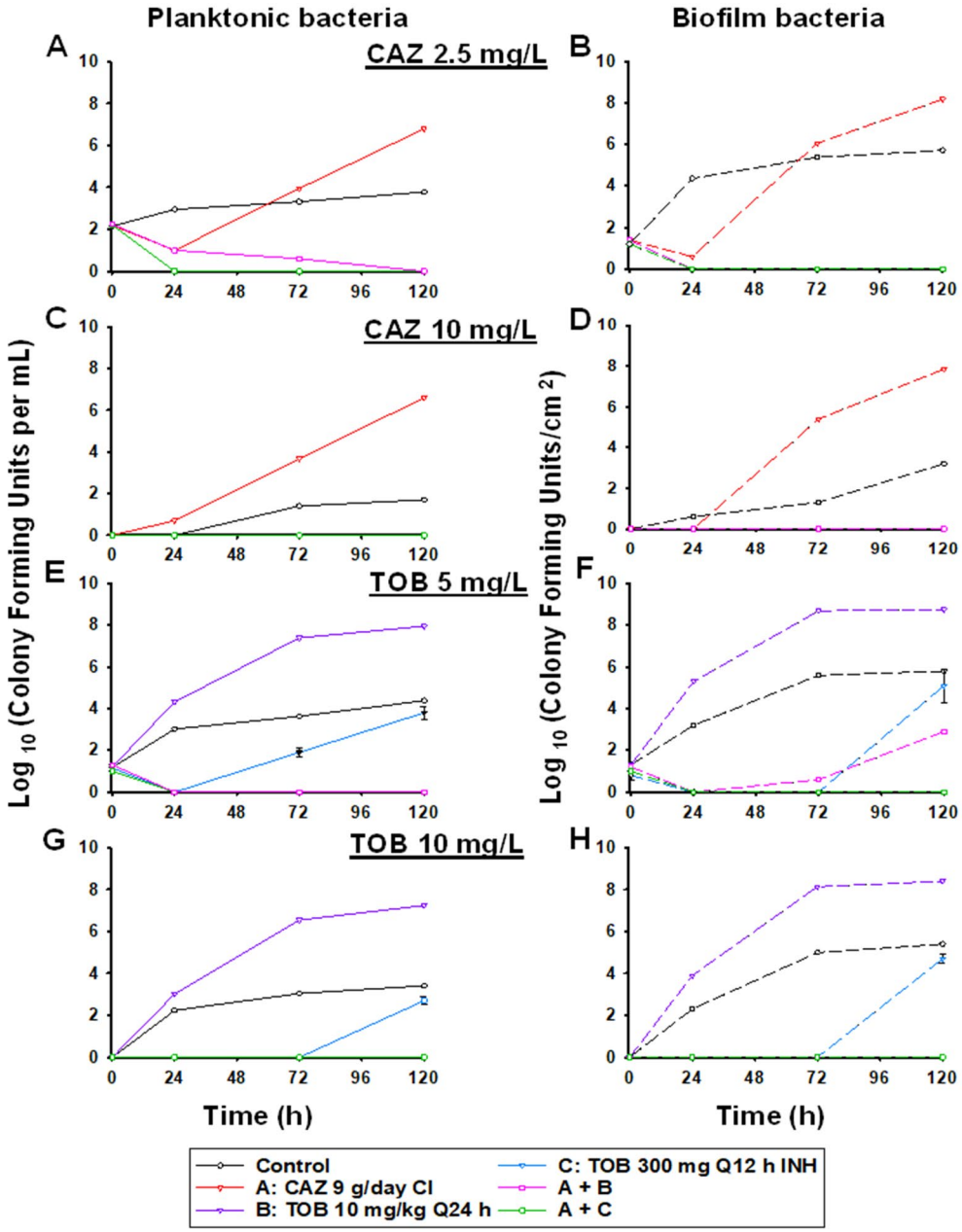
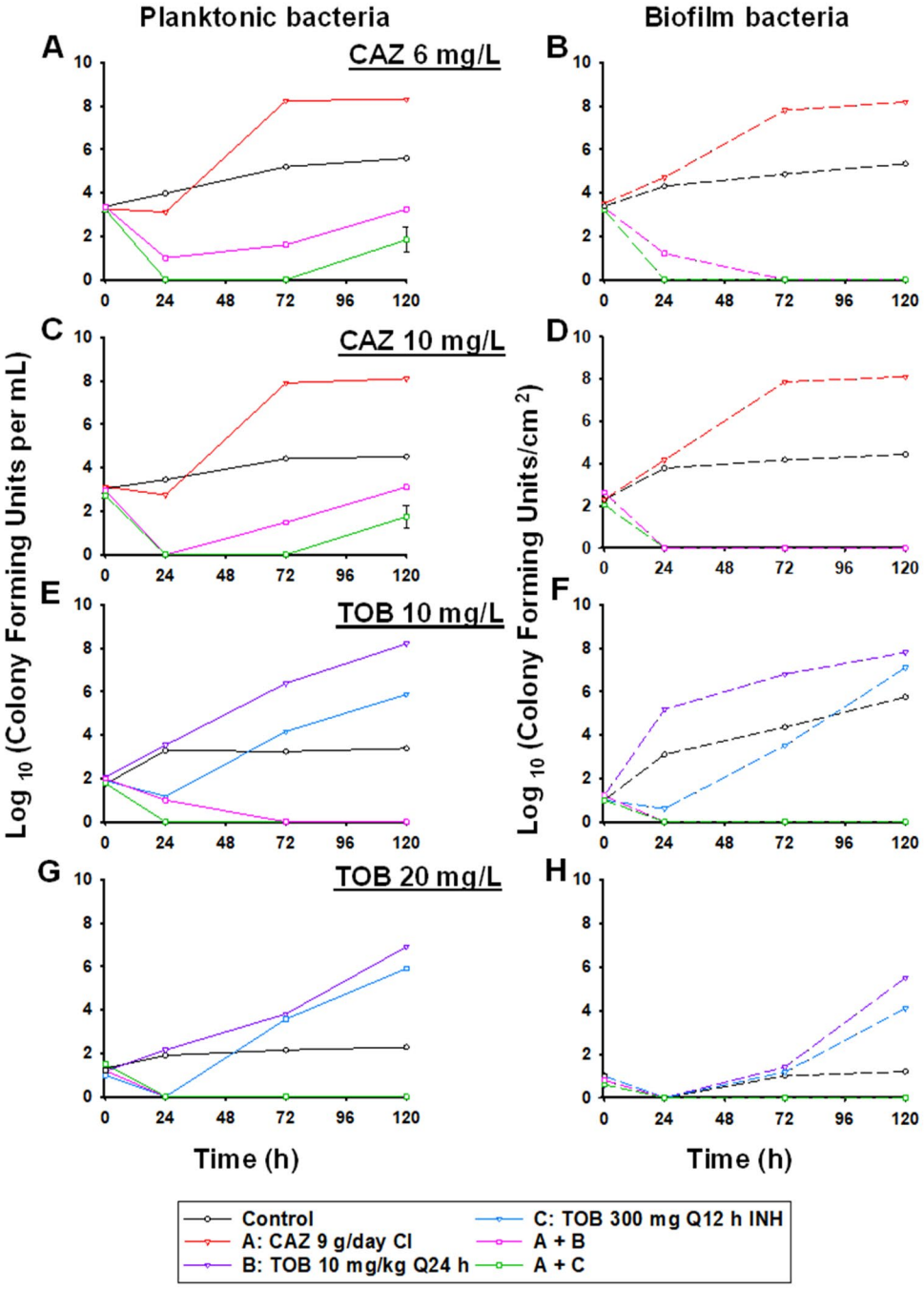


Figure 2. Effect of each dosage regimen on the counts of CW30 able to grow on agar plates containing 2.5 or 10 mg/L of ceftazidime and 5 or 10 mg/L of tobramycin. The results for treatment arm C are presented as average ± SE of two replicates; treatment arm A + C was also conducted in two replicates.



To differentiate less-susceptible subpopulations from the predominant population, the antibiotic concentrations in agar were based upon Etest MICs which were 0.064 mg/L for ceftazidime and 0.75 mg/L for tobramycin.[26]

Figure 3. Effect of each dosage regimen on the CW8 counts able to grow on agar plates containing 6 or 10 mg/L of ceftazidime, and 10 or 20 mg/L of tobramycin. The results from treatment arm A + C are presented as average ± SE of two replicates.



To differentiate less-susceptible subpopulations from the predominant population, the antibiotic concentrations in agar were based upon Etest MICs which were 0.75 mg/L for ceftazidime and 0.75 mg/L for tobramycin.[26]

Table 1. Clinically representative lung fluid concentrations, exposures and pharmacokinetic/pharmacodynamic indices for ceftazidime and/or tobramycin against CW30 and CW8 in the CBR.

Isolate	Treatment	fC_{ss} fC_{max}/fC_{min} (mg/L)	$fAUC_{24}$ (mg·h/L)	fC_{ss}/MIC fC_{max}/MIC fC_{min}/MIC	$fT_{>MIC}$ (%)	$fAUC_{24}$/MIC
CW30	CAZ 9 g/day CI	24	576	48	100	1,152
	TOB 10 mg/kg Q24 h	12.3/0.1	64.4	6.1/0	-	32.2
	TOB 300 mg Q12 h INH	100/9.4	928	50/4.7	-	464
CW8	CAZ 9 g/day CI	24	576	12	100	288
	TOB 10 mg/kg Q24 h	12.3/0.1	64.4	1.5/0	-	8.1
	TOB 300 mg Q12 h INH	100/9.4	928	12.5/1.2	-	116

CAZ, ceftazidime; TOB, tobramycin; CI, continuous infusion; INH, inhalation; fC_{ss}, unbound steady-state concentration; fC_{max}, unbound peak concentration; fC_{min}, unbound minimum concentration before next dose; $fAUC_{24}$, the area under the unbound concentration-time curve over 24 h; fC_{max}/MIC, the ratio of fC_{max} to MIC; $fT_{>MIC}$, the cumulative percentage of a 24 h period that unbound concentrations exceeded the MIC; $fAUC_{24}$/MIC, the ratio of $fAUC_{24}$ to MIC.

The simulated half-life was 3.5 h for tobramycin.

The simulated lung fluid penetration was 33% for ceftazidime, and was 50% for tobramycin.

The simulated dose of tobramycin was intravenous, unless specified as inhalation (INH).

Table 2. Log changes in viable-cell counts of total bacteria at various time points with clinically relevant lung fluid concentration exposures of ceftazidime and/or tobramycin.

Isolate	Time (h)	Log change= [$\log_{10}(CFU_t) - \log_{10}(CFU_0)$]									
		A: CAZ 9 g/day CI		B: TOB 10 mg/kg Q24h		C: TOB 300 mg Q12h INH		A + B		A + C	
		Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria
CW30	**1**	-1.24		-1.32		-2.47		-2.24		-3.48	
	3	-2.04	-0.58	-1.79	-0.34	-3.49	-0.49	-2.67	-0.62	-3.87	-0.84
	5	-2.64		-1.92		-4.02		-3.22		-4.98	
	7	-2.90	-0.69	-2.05	-0.61	-4.19	-0.73	-3.24	-0.79	-5.37	-1.22
	24	-3.53	-1.09	0.18	0.60	-3.92	-1.53	-3.72	-2.11	-5.05	-2.33
	28	-3.85		0.15		-4.95		-3.52		-7.50	
	48	-3.24	-1.80	0.78	0.49	-3.59	-2.55	-3.49	-3.17	-5.30	-4.75
	72	-2.40	-1.08	1.06	0.74	-2.87	-3.57	-3.80	-3.40	-4.78	-4.82
	96	-1.55	-0.90	1.21	0.78	-2.98	-3.58	-4.10	-3.90	-7.50	-6.93
	120	-0.41	0.03	1.16	0.77	-2.84	-1.70	-4.40	-4.10	-6.73	-6.86
CW8	**1**	-1.61		-1.85		-2.42		-1.98		-3.45	
	3	-1.73	-0.49	-2.00	-0.03	-2.94	-0.15	-2.77	-0.53	-3.49	-0.65
	5	-1.88		-2.32		-3.05		-3.34		-4.09	
	7	-2.25	-0.59	-2.28	-0.22	-2.72	-0.30	-3.16	-0.46	-3.65	-1.24
	24	-3.06	-0.33	-0.92	0.51	-2.67	-0.17	-3.36	-1.38	-3.76	-2.08
	28	-2.80		-1.06		-3.61		-3.81		-5.24	
	48	-0.45	-0.55	1.06	0.94	-2.75	-0.68	-3.73	-1.61	-4.20	-3.04
	72	1.12	0.41	1.22	1.10	-2.03	-1.23	-3.30	-2.21	-3.41	-3.78
	96	1.10	0.86	1.27	1.18	-1.80	-0.63	-3.36	-1.63	-3.50	-3.30
	120	1.00	0.79	1.26	1.08	-1.28	-0.24	-3.14	-1.73	-3.58	-3.03

CAZ, ceftazidime; TOB, tobramycin. The green background indicates synergy (a ≥2-$\log_{10}$ decrease in the CFU/mL or CFU/cm^2 with the combination compared to its most active component and, for planktonic bacteria, a ≥2-$\log_{10}$ decrease in the CFU/mL compared to the initial inoculum); the blue background indicates a 1.0- to <2-$\log_{10}$ decrease in the number of CFU/mL or CFU/cm^2 with the combination compared to its most active component.

Table 3. Log_{10} mutant frequencies at 2.5 or 6 mg/L and 10 mg/L ceftazidime and 5 or 10 mg/L and 10 or 20 mg/L tobramycin.

			CEFTAZIDIME				TOBRAMYCIN			
			2.5 mg/L		10 mg/L		5 mg/L		10 mg/L	
Isolate	Time (h)	Arm	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria	Planktonic bacteria	Biofilm bacteria
CW30	0	All Regimens	-5.35	-7.10	-7.50	-8.30	-6.32	-7.00	-7.50	-8.30
	24	Control	-5.89	-4.57	-8.84	-8.33	-5.82	-5.73	-6.60	-6.63
		A: CAZ 9 g/day CI	-2.97	-6.61	-3.27	-7.21				
		B: TOB 10 mg/kg Q24 h					-3.36	-3.60	-4.66	-5.00
		C: TOB 300 mg Q12 h INH					-3.58	-6.77	-3.58	-6.77
		A + B	-2.80	-6.19	-3.80	-6.19	-3.80	-6.19	-3.80	-6.19
		A + C	-2.45	-5.98	-2.45	-5.98	-2.45	-5.98	-2.45	-5.98
	72	Control	-5.34	-3.78	-7.26	-7.87	-5.03	-3.57	-5.62	-4.17
		A: CAZ 9 g/day CI	-1.15	-1.16	-1.42	-1.82				
		B: TOB 10 mg/kg Q24 h					-1.17	-0.35	-2.01	-0.90
		C: TOB 300 mg Q12 h INH					-2.73	-4.73	-4.63	-4.73
		A + B	-3.10	-4.90	-3.70	-4.90	-3.70	-4.30	-3.70	-4.90
		A + C	-2.72	-3.48	-2.72	-3.48	-2.72	-3.48	-2.72	-3.48
	120	Control	-5.08	-3.63	-7.16	-6.15	-4.48	-3.55	-5.45	-3.95
		A: CAZ 9 g/day CI	-0.29	-0.15	-0.49	-0.48				
		B: TOB 10 mg/kg Q24 h					-0.71	-0.32	-1.42	-0.67
		C: TOB 300 mg Q12 h INH					-0.86	-1.50	-2.36	-1.90
		A + B	-3.10	-4.20	-3.10	-4.20	-3.10	-1.30	-3.10	-4.20
		A + C	-0.77	-1.45	-0.77	-1.45	-0.77	-1.45	-0.77	-1.45
			6 mg/L		10 mg/L		10 mg/L		20 mg/L	
CW8	0	All Regimens	-4.00	-4.31	-4.13	-5.28	-3.89	-4.20	-4.22	-5.29
	24	Control	-4.39	-4.63	-4.95	-8.48	-4.48	-4.18	-5.00	-4.70
		A: CAZ 9 g/day CI	-1.09	-2.54	-1.46	-3.08				
		B: TOB 10 mg/kg Q24 h					-2.81	-2.91	-4.19	-8.09
		C: TOB 300 mg Q12 h INH					-3.42	-6.81	-4.59	-7.41
		A + B	-2.90	-6.20	-3.90	-6.20	-2.90	-5.00	-3.90	-6.20
		A + C	-3.50	-5.51	-3.50	-5.51	-3.50	-5.51	-3.50	-5.51
	72	Control	-5.39	-4.48	-4.17	-4.21	-3.43	-3.98	-4.22	-4.67
		A: CAZ 9 g/day CI	-0.44	0.25	-0.55	-0.40				
		B: TOB 10 mg/kg Q24 h					-1.89	-1.88	-4.67	-7.28
		C: TOB 300 mg Q12 h INH					-1.07	-2.94	-1.66	-5.28
		A + B	-3.96	-5.34	-2.86	-5.34	-2.36	-5.34	-2.48	-5.34
		A + C	-3.85	-3.81	-3.85	-3.81	-3.85	-3.81	-3.85	-3.81
	120	Control	-5.37	-3.24	-3.67	-3.60	-3.16	-3.65	-4.24	-4.56
		A: CAZ 9 g/day CI	0.04	-0.19	-0.16	-0.25				
		B: TOB 10 mg/kg Q24 h					-0.35	-0.86	-1.62	-3.16
		C: TOB 300 mg Q12 h INH					-0.43	-0.63	-1.40	-1.60
		A + B	-4.12	-0.45	-4.12	-5.85	-0.21	-0.57	-1.01	-5.85
		A + C	-3.68	-4.55	-3.68	-4.55	-1.85	-4.55	-2.44	-4.55

CAZ, ceftazidime; TOB, tobramycin. The red background indicates a high mutant frequency, *i.e.* a large proportion of less-susceptible bacteria being present in the total population; the green background indicates a low mutant frequency, *i.e.* a small proportion of less-susceptible bacteria being present in the total population.

Chapter 6

Conclusions and future directions

Conclusions and future directions

Infections caused by Gram-negative bacteria such as *P. aeruginosa* have emerged as a major threat to global health and have been associated with increased morbidity and mortality in critically ill and CF patients. The abundant and inappropriate use of broad-spectrum antibiotics has contributed to the emergence of MDR bacteria. This has created a vicious cycle to force us to rely on additional broad-spectrum antibiotics to treat infections, leading to yet more resistance. The emergence and proliferation of these highly resistant bacteria are particularly concerning given the limited number of antibacterial agents that are currently available or in the drug development pipelines of the pharmaceutical industry. Especially concerning is the prevalence of life-threatening infections caused by *P. aeruginosa* in critically ill patients and of acute infective exacerbations caused by hypermutable biofilm-forming *P. aeruginosa* in CF patients. To address the clinical need presented by these difficult-to-manage infections, substantial efforts are required to maximise the efficacy and minimise the emergence of resistance of existing antipseudomonal antibiotics. In addressing **Hypotheses 1–4** and **Aims 1–4**, this thesis aimed: firstly, to identify and define quantitatively key PK/PD characteristics of fosfomycin against *P. aeruginosa* (Chapter 2); and, secondly, to examine and evaluate approaches to maximise the antibacterial activity of combinations of antipseudomonal antibiotics against the planktonic and biofilm growth forms of hypermutable *P. aeruginosa* (Chapters 3, 4 and 5).

PK/PD related information is an essential tool in designing optimised dosing regimens of fosfomycin using exposure-response relationships. As fosfomycin came into clinical use before the advent of contemporary drug development procedures, a dearth of knowledge about its PK and PD properties has limited its use other than for uncomplicated urinary tract infections. In addressing **Hypothesis 1 and Aim 1**, the study undertaken in Chapter 2 was specifically designed to determine the PK/PD index most closely related to antibacterial activity of fosfomycin against *P. aeruginosa* and quantify the magnitude of the predictive PK/PD index required to achieve various extents of bacterial killing or needed to prevent the emergence or

amplification of fosfomycin-resistant subpopulations. A dynamic *in vitro* PK/PD infection model was used to identify the PK/PD index, *i.e.* $fT_{>MIC}$, fAUC/MIC, or fC_{max}/MIC that best predicts fosfomycin efficacy. Dose fractionation was conducted over 24 h against three strains of *P. aeruginosa* (one reference strain; ATCC 27853, and two MDR clinical isolates; CR1005, CW7). A large range of fosfomycin concentrations (fC_{max} range: 6.25 – 3000 mg/L) and 30 different dosing regimens were simulated to maximally differentiate among the PK/PD indices under investigation. For fosfomycin the fAUC/MIC was the PK/PD index most closely correlated with its efficacy against *P. aeruginosa*. Although it had been previously reported that bacterial killing by fosfomycin against this organism was time-dependent, we were unable to find a relationship between activity and $fT_{>1\times MIC}$. This may be explained by a large number of the dosing regimens having a $fT_{>1\times MIC}$ of 100%. To account for this, we also analysed each index at 10×MIC. Although a relationship was present between antibacterial activity and $fT_{>10\times MIC}$, activity was most closely associated with fAUC/(10×MIC), indicating the importance of time-averaged exposure to fosfomycin. Values of fAUC/MIC required to achieve various magnitudes of killing effect against the reference strain were subsequently determined. Bacterial killing of no more than ~3-$\log_{10}$ CFU/mL was achieved with any regimen against any isolate. The proposed fAUC/MIC targets required to achieve 1- and 2-$\log_{10}$ reductions in the area under the CFU/mL *versus* time curve relative to growth control were 489 and 1024, respectively. Irrespective of the fC_{max}/MIC, fAUC/MIC and $fT_{>MIC}$ values achieved, substantial regrowth occurred by 24 h with all regimens. None of the regimens was able to suppress the emergence of fosfomycin resistance.

The study described in Chapter 2 was the first to utilise an extensive dose-ranging and dose-fractionation design to identify the most predictive PK/PD index for fosfomycin activity against *P. aeruginosa* isolates in an *in vitro* PK/PD model. In the future, additional studies in a number of other areas will be beneficial to support this study. Firstly, *in vivo* dose-ranging, dose-fractionation PK/PD studies are warranted in different animal models, with different infection sites, to assess the modulatory effects of the immune system on residual populations. Secondly, multi-omic (genomic, transcriptomic, metabolomic) analysis of parent and emergent

resistant populations would assist in identifying molecular mechanisms involved in adaptation and amplification of fosfomycin resistance. Information about these mechanisms will assist our understanding of how resistance to fosfomycin develops over time and provide beneficial insights for the designing of effective dosing strategies. Thirdly, as our data suggest that monotherapy with fosfomycin may be problematic for the treatment of infections caused by *P. aeruginosa* due to emergence of resistance, systematic investigation of combinations of fosfomycin with other antibiotics may be beneficial in combating *P. aeruginosa* infections. Studies examining the time-course of antibacterial effects of fosfomycin combinations should be conducted in dynamic models (*in vitro* and *in vivo* PK/PD) and, when studying biofilm-forming strains, investigate effects on biofilm structure using confocal imaging and other techniques. Finally, all of this information will assist in developing novel next-generation *in silico* mechanism-based mathematical models for the exploration of antibacterial effects of different dosage regimens of fosfomycin in different patient populations to suggest optimised and individualised dosing schemes for translation to, and evaluation in, the clinic.

Given the resilience of hypermutable *P. aeruginosa* due to increased mutation rate and the ability to form biofilm, and the insufficiency of current antibiotic dosing regimens to effectively kill and suppress the regrowth and resistance, the antibacterial activities of important antipseudomonal antibiotics in monotherapy and in different combinations were examined in Chapter 3-5 to test **Hypotheses 2-4 and Aims 2-4**. Hypermutable isolates were investigated in the dynamic *in vitro* CBR model over 120 h. Antibiotics with different mechanisms of action and resistance were selected for each of the combinations examined. Concentration-time profiles of antibiotics clinically achievable in the lung fluid of patients with CF were simulated. Viability counts of total and less-susceptible planktonic and biofilm bacteria were quantified at various time points.

In Chapter 3, we systematically characterised for the first time the effect of simulated lung fluid concentration-time profiles of a standard meropenem regimen (*i.e.* intermittent 30-min infusions every 8 h) *versus* a modified meropenem regimen (*i.e.* a constant-rate continuous infusion), for delivery of the same daily dose, without or with tobramycin, against a

carbapenem-resistant MDR hypermutable clinical isolate. Because of clinical reports of different levels of penetration of meropenem into lung fluid after intravenous administration (30% and 60%), these two levels of penetration were simulated with continuous infusion regimens of meropenem. The standard regimen of meropenem, both as monotherapy and in combination, was ineffective in suppressing regrowth and emergence of resistance against both planktonic and biofilm bacteria. The combination with the modified meropenem regimen not only demonstrated synergistic or enhanced bacterial killing but also suppressed regrowth of less susceptible bacteria, even under the conditions simulating the low level of lung fluid penetration (30%) of meropenem. The combination with meropenem administered as a continuous infusion at 60% lung fluid penetration in addition synergistically suppressed regrowth of total biofilm bacteria. It is not surprising that the combination with standard meropenem dosing was less effective than that with meropenem delivered as a continuous infusion, as in the former case there were substantial periods with essentially no antibiotic present for activity. This study was conducted over five days of treatment, quantified both biofilm and planktonic bacteria and evaluated emergence of resistance. In addition, multiple biological replicates were performed to demonstrate reproducibility of the total and less-susceptible bacterial counts. Possible future studies are discussed below.

In Chapter 4, we prospectively evaluated the impact of simulated clinically relevant lung fluid PK profiles of intravenous ciprofloxacin and meropenem, as monotherapy and in combination, against one hypermutable *P. aeruginosa* reference strain (PAOΔ*mutS*) and one hypermutable clinical isolate in the dynamic *in vitro* CBR model over 120 h. Ciprofloxacin was administered as 1-h infusions every 8 h; while meropenem was administered as a continuous infusion, with two levels of penetration into lung fluid (30% and 60%) examined. Our results showed that neither ciprofloxacin nor meropenem were effective in monotherapy against planktonic and biofilm bacteria of hypermutable *P. aeruginosa* strains. However, both antibiotics in combination regimens demonstrated very promising results at simulated patient PK profiles achievable with FDA-approved daily doses in patients with CF. The combination regimens not only exhibited synergistic bacterial killing and resistance suppression against planktonic

bacteria but also against biofilm bacteria for both isolates. The combination with meropenem at lung fluid concentrations simulating the high level of penetration also suppressed amplification of resistance of biofilm bacteria for the hypermutable *P. aeruginosa* reference strain. This is the only *in vitro* biofilm study to explore the effects of concentration-time profiles representative of those in lung fluid for the ciprofloxacin-meropenem combination against planktonic and biofilm bacteria of hypermutable *P. aeruginosa* strains. Possible future studies are discussed below.

In Chapter 5, we systematically investigated the effect of clinically relevant lung fluid concentration-time profiles of ceftazidime, administered intravenously as a continuous infusion, and tobramycin (administered intravenously every 24 h *versus* as an inhalation every 12 h), as monotherapy and in combination, on bacterial killing and resistance emergence of hypermutable *P. aeruginosa*. The time-course of bacterial killing and resistance emergence/suppression of both planktonic and biofilm bacteria was examined over 120 h in the CBR against two hypermutable *P. aeruginosa* clinical isolates. This study demonstrated that as monotherapy neither antibiotic in a regimen simulating intravenous administration was able to provide sustained reduction in total and resistant subpopulations of both planktonic and biofilm bacteria. The monotherapy regimen of tobramycin simulating administration by inhalation provided substantially greater antibacterial effect than when the same drug was administered in the simulated intravenous regimen. Both combination treatments demonstrated synergistic bacterial killing, not only for planktonic but also biofilm bacteria; however, greatest bacterial killing against both modes of bacterial growth was observed with the combination simulating tobramycin inhalation. This is the first study to examine the activity of clinically relevant regimens of ceftazidime and tobramycin in monotherapy and in combination against isolates of *P. aeruginosa* that were hypermutable, representing a worst-case scenario. Other notable features of the study include: the impact of delivering tobramycin by inhalation *versus* intermittent intravenous infusion was investigated; the effects on both planktonic and biofilm bacteria were examined in a dynamic model over 5 days; and, the time-

courses of both total and resistant subpopulations in response to the monotherapy and combination regimens were characterised.

The studies described in Chapters 3, 4 and 5 in which the antibacterial activities of antipseudomonal antibiotics in monotherapy and combination regimens were examined against planktonic and biofilm bacteria of hypermutable *P. aeruginosa* strains have been very informative. The studies demonstrated that combining antibiotics with different mechanisms of action and resistance is superior to monotherapy in combating difficult-to-treat hypermutable *P. aeruginosa* strains. The results of the studies consistently support a key recommendation of CF treatment guidelines, that antibiotics should not be used in monotherapy regimens for treatment of acute pulmonary infective exacerbations. The studies demonstrated the very substantial synergistic or enhanced effect that can be achieved with combination regimens, especially when regimens are modified to improve exposure with intravenous administration or, in the case of tobramycin, the drug is administered in a regimen simulating inhalation.

The highly encouraging results of the research reported in Chapters 3, 4 and 5 point to a number of possible future studies. Firstly, as we have demonstrated that modifying the mode of intravenous administration of β-lactam antibiotics (*i.e.* intermittent *versus* continuous infusion) can markedly affect activity against hypermutable *P. aeruginosa*, additional studies with prolonged infusions (3-4 h) of β-lactams in combination with front-loaded dosing regimens of tobramycin or ciprofloxacin may be beneficial to maximise bacterial killing and suppress the amplification of less-susceptible subpopulations. Secondly, further studies could investigate the effects of sequencing of anti-pseudomonals in combination in the CBR, such as starting the treatment with a concentration-dependent, rapidly-killing antibiotic to substantially decrease the inoculum followed by the administration of a second antibiotic for the early eradication of *P. aeruginosa*. As noted above, the studies in Chapters 3, 4 and 5 have demonstrated that combining antibiotics with different mechanisms of bacterial killing and resistance can combat hypermutable *P. aeruginosa* in the CBR. As hypermutable *P. aeruginosa* isolates from CF patients have increased mutation rate and easily become MDR, for isolates where resistance is believed to be mediated by a β-lactamase the evaluation of

activity of antibiotic preparations, *e.g.* ceftolozane-tazobactam, ceftazidime-avibactam, meropenem-vaborbactam and imipenem-cilastin-relebactam could be beneficial. Other strategies, such as including in a combination an agent that impedes the development of biofilm and/or disrupts already established biofilm (*e.g.* azithromycin which affects quorum sensing, *N*-acetyl cysteine which disrupts extracellular polymeric substance) should also be considered. In the various CBR studies outlined above, confocal imaging of biofilm structure and multi-omic analysis of planktonic and biofilm bacteria would provide more detailed insights to better understand the pattern of bacterial killing and resistance emergence. Although the CBR is an ideal *in vitro* dynamic PK/PD model for simulating PK profiles as occur in patients and examining antibacterial effects, *in vivo* studies in animal models would enable the assessment of immune system effects on residual populations.

All of the important information generated from the above-mentioned preclinical studies will assist in developing novel next-generation mechanism-based models for the optimisation of combination regimens of antibiotics, and possibly ancillary agents, for translation to the clinic for evaluation in appropriate clinical trials. The promising results achieved with the regimen simulating inhalational delivery of tobramycin (Chapter 5) also reinforce calls for appropriate clinical studies to investigate the administration of antibiotics by inhalation for the treatment of acute pulmonary infective exacerbations.

In conclusion, the research described in this thesis provides important new information relating to the antimicrobial pharmacology of fosfomycin for treatment of infections caused by *P. aeruginosa* and the use of combination regimens of antibiotics for management of acute infective exacerbations caused by hypermutable, biofilm-forming strains of that pathogen in people with CF.

Appendix

Appreciation Letter (Editor General, *JAC*) Chapter 2

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Comment on: Elucidation of the pharmacokinetic/pharmacodynamic determinants of fosfomycin activity against *Pseudomonas aeruginosa* using a dynamic *in vitro* model

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Sir,

Bilal *et al.*[1] are to be applauded for elucidating the low barrier to resistance afforded by *Pseudomonas aeruginosa*, when confronted by treatment with fosfomycin. Whilst fosfomycin is an advantageous addition to our antimicrobial armamentarium, thanks to its activity against otherwise MDR strains of Enterobacterales, its role in the *in vivo* treatment of *P. aeruginosa* infections is far from clear, yet frequently cited.[2]

Studies describing fosfomycin's use as monotherapy or combined therapy for MDR *P. aeruginosa* infections are flawed by low case numbers, heterogeneous combination therapy, variability in susceptibility testing and a lack of control arms – the latter being an all too frequent encounter in the management of MDR pathogens.[3] The method and interpretation of susceptibility testing is particularly challenging. A systematic review by Falagas and colleagues[3] cites a 30.2% susceptibility rate of MDR *P. aeruginosa* to fosfomycin, however, data from the individual studies reveals vast heterogeneity in the results of various susceptibility testing methods. Broth microdilution, agar dilution, disc diffusion and gradient strip methods each respectively had a median susceptibility of 91.1% (mean 58.1%, range 0%-100%, SD±45%), 90% (mean 70%, range 0%-100%, SD±41%), 56.3% (mean 51%, range 0%-100%, SD±35) and 11.1% (mean 28.6%, range 0%-93.3%, SD±35%).[3] This mirrors our local

experience, where a diverse population [according to variable number tandem repeat (VNTR) profiling] of 13 clinical isolates of *P. aeruginosa* was assessed for susceptibility to fosfomycin by disc diffusion, automated susceptibility testing (AST; BD Phoenix) and gradient strip (Etest). 7.6% were susceptible by disc diffusion, 53.8% by AST and 15.3% by Etest. With this degree of heterogeneity, how can a routine clinical microbiology laboratory be confident what constitutes a susceptible strain of *P. aeruginosa*?

Whilst fosfomycin reaches its active site in Enterobacterales via two permeases, encoded by the *glpT* and *uhpT* genes, in *P. aeruginosa*, *uhpT* is redundant, meaning resistance is acquired by a single mutational step, which affords no disadvantage to fitness.[4] Bilal *et al*.[1] clearly demonstrate that even concentrations of fosfomycin greater than those clinically achievable *in vivo* were associated with rapid emergence of *P. aeruginosa* resistance, probably due to resistant subpopulations present at the beginning of treatment. The same warning is sounded by Docobo-Pérez *et al*.,[5] whose *in vitro* model suggests rapid development of fosfomycin resistance in ESBL-producing *Escherichia coli*, exposed to the equivalent of *in vivo* doses of fosfomycin below 24 g/day.

In spite of these concerns, good outcomes in respiratory function have been observed when fosfomycin was used therapeutically in cases of MDR *P. aeruginosa* pulmonary infection in patients with underlying cystic fibrosis.[6] However, this is in the setting of combination therapy with a known antipseudomonal antimicrobial, and interestingly was not always associated with confirmatory susceptibility testing of the infecting strains to fosfomycin.[6] This raises several questions: should susceptibility testing be in association with another antimicrobial, such as a fluoroquinolone or aminoglycoside; does fosfomycin have a mechanism of action regardless of *in vitro* susceptibility testing; or does it have additional anti-inflammatory properties?

With multiple outstanding questions, including which method of susceptibility testing to use, choice of combination therapy, infective diagnosis, dosage, etc., fosfomycin is far from being recognized as an antipseudomonal antimicrobial. Research in the setting of large,

randomized controlled trials is welcomed, before there is any further overuse of this antibiotic against a pathogen for which it simply cannot compete.

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Transparency declarations

None to declare.

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