Fighting resistant bacterial 'superbugs' by novel antibiotic combination dosing strategies

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This thesis is dedicated to
my dearest parents, Jillian and Murray,
and my loving Grandma

Table of Contents

ABSTRACT ..... **iv**
THESIS INCLUDING PUBLISHED WORKS DECLARATION ..... **vi**
GENERAL DECLARATION ..... **viii**
PUBLICATIONS DURING ENROLMENT ..... **ix**
CONFERENCE PRESENTATIONS DURING ENROLMENT ..... **ix**
ACKNOWLEDGEMENTS ..... **xii**
LIST OF ABBREVIATIONS ..... **xiv**
CHAPTER ONE: INTRODUCTION **1**
1.1 Overview 2
1.2 Antibacterial resistance crisis 3
1.3 Cystic fibrosis 5
1.3.1 *P. aeruginosa* prevalence and impact in respiratory disease 6
1.4 Hypermutable *P. aeruginosa* 7
1.5 Antipseudomonal agents and resistance 11
1.5.1 Aminoglycosides 12
1.5.2 Beta-lactam antibiotics 14
1.5.3 Fluoroquinolones 15
1.5.4 The impact of hypermutation on the susceptibility to antimicrobial agents 16
1.6 Approaches to antimicrobial treatment 17
1.6.1 Pharmacokinetic/pharmacodynamic indices 19
1.6.2 *In silico* evaluation 21
1.6.3 Antimicrobial combination therapy 22
1.6.4 Antipseudomonals against hypermutators 24
1.7 Summary 30
1.8 Statement of hypotheses and aims 31
1.8.1 Hypotheses 31
1.8.2 Project Aims 31
1.9 Thesis Layout 32
1.10 References 33

CHAPTER TWO: SHAPE DOES MATTER: SHORT HIGH-CONCENTRATION EXPOSURE MINIMIZES RESISTANCE EMERGENCE FOR FLUOROQUINOLONES IN *PSEUDOMONAS AERUGINOSA* 47

Preamble 48

Published journal article 49

CHAPTER THREE: RESISTANCE SUPPRESSION BY HIGH-INTENSITY, SHORT-DURATION AMINOGLYCOSIDE EXPOSURE AGAINST HYPERMUTABLE AND NON-HYPERMUTABLE *PSEUDOMONAS AERUGINOSA* 58

Preamble 59

Published journal article 60

Supplementary material 71

CHAPTER FOUR: IMPACT OF HYPERMUTATION ON THE EMERGENCE OF RESISTANCE OF *PSEUDOMONAS AERUGINOSA* EXPOSED TO CLINICALLY RELEVANT CONCENTRATIONS OF DIVERSE ANTIBIOTICS 72

Preamble 73

Submitted manuscript 74

Abstract 75

Introduction 76

Materials and methods 77

Results and discussion 79

Conclusions 84

References 85

CHAPTER FIVE: CHARACTERIZATION OF HYPERMUTATOR *PSEUDOMONAS AERUGINOSA* ISOLATES FROM PATIENTS WITH CYSTIC FIBROSIS IN AUSTRALIA 90

Preamble 91

Manuscript 92

Abstract 93

Importance 94

Introduction 95

Results and discussion 97

Materials and methods 105

References 109

Supplementary material 119

CHAPTER SIX: MEROPENEM IN COMBINATION WITH CIPROFLOXACIN COMBATS HYPERMUTABLE *PSEUDOMONAS AERUGINOSA* FROM RESPIRATORY INFECTIONS OF CYSTIC FIBROSIS PATIENTS..... 122

Preamble 123

Manuscript 124

Abstract 125

Introduction 126

Results 127

Discussion 130

Materials and Methods 133

References 139

Supplementary material 151

CHAPTER SEVEN: CONCLUSIONS AND FUTURE DIRECTIONS 162

Abstract

Antimicrobial resistance poses one of the greatest threats to human health worldwide with fears of a return to the pre-antibiotic era. The suboptimal use of antibiotics has contributed to this rise in antimicrobial resistance. This global threat is exacerbated by dwindling research into antibiotic development and the lack of effective monotherapies against bacterial 'superbugs.' Pharmacokinetic/pharmacodynamic (PK/PD) indices are used to aid the choice of dosage regimens, however limitations to these indices require investigation. For cystic fibrosis (CF) patients this threat is ever present, facing frequent respiratory infections by bacterial pathogens, due to mucus overproduction creating a favourable growth environment. CF patients often require lengthy antibiotic treatments that usually result in the development of resistance. Respiratory infections due to *Pseudomonas aeruginosa* have been found to be a main driver of mortality. Furthermore, *P. aeruginosa* from chronic respiratory infections often exhibit hypermutator phenotype, where an increased mutation rate allows these hypermutators to gain or enhance their mechanisms of resistance. The prevalence of hypermutators in Europe has been well documented, however this has not been broadly assessed in an Australian clinic. Only a few studies have assessed antibiotic therapies against hypermutable *P. aeruginosa* and most have resulted in emergence of resistance, suggesting that monotherapy is likely not a viable choice against infections caused by such strains. Hence, there is an urgent need for research into rational combination treatment regimens. This thesis combines PK/PD index evaluation, static and dynamic *in vitro* studies, bioinformatics and mechanism-based modelling to characterise the antibacterial effects of clinically important antibiotics in mono- and combination therapy. Both hypermutable and non-hypermutable *P. aeruginosa* were examined. In addressing the aims and hypotheses of this thesis, we identified a promising, synergistic antibiotic combination against a clinical *P. aeruginosa* hypermutator, which produced substantial bacterial killing and suppression of resistance. We

also found that the shape of the concentration-time profile was important for suppression of resistance with a fluoroquinolone and an aminoglycoside. Further, we comprehensively characterised *P. aeruginosa* isolates obtained from respiratory infections of CF patients from an Australian clinic, and identified thirteen hypermutators that were further characterised at the gene level. These findings have significantly contributed to identifying clinically relevant, synergistic antibiotic combinations to fight hypermutable *P. aeruginosa* 'superbugs.' These promising combinations require further evaluation in the future to treat respiratory infections in CF patients with hypermutable *P. aeruginosa*. Ultimately, the systematic evaluation of clinically important antibiotics against hypermutators in this thesis contributes to the optimisation of rational antibiotic combination dosage regimens for CF patients infected with *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 two original research papers published in and one original manuscript submitted for publication to peer-reviewed journals. Two manuscripts are ready for submission (under internal review with co-authors) to a peer-reviewed journal for publication. The core theme of the thesis is establishing the impact of bacterial hypermutators and evaluating antibiotic combination dosage regimens. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Centre for Medicine Use and Safety, Faculty of Pharmacy and Pharmaceutical Sciences, Monash University under the supervision of Dr. Cornelia B. Landersdorfer.

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

In the case of Chapters 2 to 4 my contribution to the work involved the following:

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	Shape does matter: short high-concentration exposure minimizes resistance emergence for fluoroquinolones in *Pseudomonas aeruginosa*	Published *JAC 2015; 70:818-26*	70%. Experimental design, execution, authorship, preparation of figures, tables and first draft of manuscript	1) Jürgen B. Bulitta, Joint senior authorship, experimental design and editorial review 10% 2) Roger L. Nation, Editorial review 5% 3) Brian T. Tsuji, Editorial review 2.5% 4) Fritz Sörgel, Editorial review 2.5% 5) Cornelia B. Landersdorfer, Senior/corresponding author, experimental design and editorial review 10%	No No No No No
3	Resistance suppression by high-intensity, short-duration aminoglycoside exposure against hypermutable and non-hypermutable *Pseudomonas aeruginosa*	Published *JAC 2016; 71:3157-67*	70%. Experimental design, execution, mathematical modelling, authorship, preparation of figures, tables and first draft of manuscript	1) Jürgen B. Bulitta, Joint senior authorship, mathematical modelling assistance and editorial review 7.5% 2) Antonio Oliver, Supplied isolate and editorial review 5% 3) Brian T. Tsuji, Editorial review 2.5% 4) Craig R. Rayner, Editorial review 2.5% 5) Roger L. Nation, Editorial review 5% 6) Cornelia B. Landersdorfer, Senior/corresponding author, experimental design, mathematical modelling assistance and editorial review 7.5%	No No No No No No
4	Impact of hypermutation on the emergence of resistance of *Pseudomonas aeruginosa* exposed to clinically relevant concentrations of diverse antibiotics	Submitted *IJAA-S-17-01590*	80%. Experimental design, execution, authorship, preparation of figures, tables and first draft of manuscript	1) Jürgen B. Bulitta, Editorial review 2.5% 2) Antonio Oliver, Supplied isolate, editorial review 2.5% 3) Roger L. Nation, Editorial review 5% 4) Cornelia B. Landersdorfer, Senior author, experimental design and editorial review 10%	No No No No

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

Student's Signature	[redacted]	**Date** 18/12/2017

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	[redacted]	**Date** 18/12/2017

General declaration

Chapters 5 and 6 contain unpublished work and these are presented similarly to submitted Chapters. These manuscripts are under internal review with co-authors, and will be submitted shortly 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	Characterisation of mutator *Pseudomonas aeruginosa* from patients with cystic fibrosis in Australia	Under internal review with co-authors	65%. Experimental design, execution, authorship, preparation of figures, tables and first draft of manuscript	1) Deanna S. Deveson Lucas, Assisted in characterisation and editorial review 7% 2) Yuling Huang, Assisted in rifampicin mutation frequencies 2% 3) Antonio Oliver, Supplied strains and editorial review 2% 4) Jürgen B. Bulitta, Editorial review 1.5% 5) Anton Y. Peleg, Made clinical isolates available and editorial review 1.5% 6) Tom Kotsimbos, Collected clinical isolates 1.5% 7) Roger L. Nation, Editorial review 2% 8) Carla López-Causapé, Editorial review 1.5% 9) Murray C. Rees, Statistical support and editorial review 2% 10) John D. Boyce, Joint senior author, assisted in characterisation and interpretation of results, editorial review 7% 11) Cornelia B. Landersdorfer, Joint senior author, experimental design and editorial review 7%	No No No No No No No No No No No
6	Meropenem in combination with ciprofloxacin combats hypermutable Pseudomonas aeruginosa from respiratory infections of cystic fibrosis patients	Under internal review with co-authors	60%. Experimental design, execution, mathematical modelling, authorship, preparation of figures, tables and first draft of manuscript	1) Rajbharan Yadav, Mathematical modelling assistance and editorial review 5% 2) Kate E. Rogers, HFIM assistance 5% 3) Jürgen B. Bulitta, Editorial review 2.5% 4) Veronika Wirth, LC-MS/MS assistance 5% 5) Antonio Oliver, Supplied strains and editorial review 5% 6) Anton Y. Peleg, Supplied isolates and editorial review 2.5% 7) Roger L. Nation, Experimental design and editorial review 5% 8) Cornelia B. Landersdorfer, Senior author, experimental design, mathematical modelling assistance and editorial review 10%	Yes No No No No No No No

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Publications during candidature

1. **Rees VE**, Bulitta JB, Nation RL, Tsuji BT, Sörgel F, Landersdorfer CB "Shape does matter: Short high-concentration exposure minimises resistance emergence for fluoroquinolones in Pseudomonas aeruginosa" J Antimicrob Chemother 2015; 70(3): 818-26.

2. **Rees VE**, Bulitta JB, Oliver A, Tsuji BT, Nation RL, Rayner CR, Landersdorfer CB "Resistance suppression by high-intensity, short-duration aminoglycoside exposure against hypermutable and non-hypermutable Pseudomonas aeruginosa" J Antimicrob Chemother 2016; 71 (11): 3157-67.

Conference presentations during enrolment

1. **Rees VE** "Optimised combination therapy: the future to eradicate hypermutable bacteria" Oral at the 27th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) conference, Vienna, Austria, 22-25th April, 2017 (#4571).

2. **Rees VE** "Optimised combination therapy: the future to eradicate hypermutable bacteria" Oral at the International Society for Anti-Infective Pharmacology (ISAP) conference, Vienna, Austria, 21st April, 2017.

3. **Rees VE**, Bulitta JB, Oliver A, Peleg AY, Kotsimbos T, Rees MC, Nation RL, Landersdorfer CB "Hypermutable Pseudomonas aeruginosa related to multi-drug resistance in cystic fibrosis patients with respiratory infections" Poster at the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists (ASCEPT) – Molecular Pharmacology of GPCRs (MPGPCR) Joint Scientific Meeting, 27-30th November, 2016 (#262).

4. **Rees VE**, Bulitta JB, Oliver A, Peleg AY, Kotsimbos T, Rees MC, Nation RL, Landersdorfer CB "Hypermutable Pseudomonas aeruginosa related to multi-drug resistance from respiratory infections in cystic fibrosis" Poster at 11th Annual Postgraduate Symposium, Monash University, 3rd October, 2016.

5. **Rees VE**, Bulitta JB, Oliver A, Sörgel F, Tsuji B, Rayner C, Nation RL, Landersdorfer CB "Mechanism-based modelling to assess suppression of bacterial resistance by high intensity, short duration tobramycin exposure" Poster at the 2nd World Conference on Pharmacometrics (WCoP), Brisbane, 21-24th August, 2016 (252).

6. **Rees VE** "Combating antibiotic resistant 'superbugs'" Oral at 3 Minute Thesis (3MT) competition, Monash University, 21st July, 2016.

7. **Rees VE**, Bulitta JB, Tsuji BT, Oliver A, Nation RL, Landersdorfer CB "Aminoglycoside Resistance Prevention by Short-Term, High Concentration Exposure in Pseudomonas aeruginosa Assessed via Novel Mechanism-Based Modeling (MBM)" Poster at the American Society for Microbiology (ASM)-MICROBE Conference, Boston, 16-20th June, 2016 (Sunday-511).

8. **Rees VE**, Bulitta JB, Peleg AY, Kotsimbos T, Nation RL, Oliver A, Landersdorfer CB "Hypermutable Pseudomonas aeruginosa Linked with Antibiotic Resistance in Australian Cystic Fibrosis Patients" Poster at the American Society for Microbiology (ASM)-MICROBE Conference, Boston, 16-20th June, 2016 (Saturday-256).

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10. **Rees VE**, Bulitta JB, Oliver A, Tsuji BT, Nation RL, Landersdorfer CB "The Concentration-Time Profile Shape Plays a Key Role in Aminoglycoside Resistance Prevention in Pseudomonas aeruginosa" Poster at the Biomed Link Conference, 20th November, 2015 (B39).

11. **Rees VE**, Bulitta JB, Nation RL, Yadav R, Rogers KE, Cheah S, Oliver A, Landersdorfer CB "Shape of the Meropenem Profile in Combination with Tobramycin is Critical to Combat Hypermutable Pseudomonas aeruginosa" Poster at the 55th Interscience Conference on Antimicrobial Agents & Chemotherapy (ICAAC), San Diego, 18-21st September, 2015 (A-1507), presented by Cornelia Landersdorfer on my behalf.

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List of abbreviations

ATM	Aztreonam
ATTC	American Type Culture Collection
AUC	Area under the concentration-time curve
CAZ	Ceftazidime
C_{min}	Minimum (or trough) concentration
CAMHA	Cation-adjusted Mueller Hinton agar
CAMHB	Cation-adjusted Mueller Hinton broth
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CFU or cfu	Colony forming units
cgSNPs	Core-genome single nucleotide polymorphisms
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
C_{max}	Maximum (or peak) concentration
ELF	Epithelial lining fluid
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
f	Free unbound
gDNA	Genomic DNA
h	hours
HFIM	Hollow fibre infection model
IPM	Imipenem
MBM	Mechanism-based modelling
MDR	Multidrug-resistant
MEM or MER	Meropenem
MF	Mutation frequencies
MIC	Minimum inhibitory concentration
MMR	Mismatch repair
NHMRC	Australian National Health and Medical Research Council
PaβN	Phenylalanine-arginine β-naphthylamide efflux pump inhibitor
PAE	Postantibiotic effect
PBP	Penicillin-binding proteins
PD	Pharmacodynamics
PK	Pharmacokinetics
PK/PD	Pharmacokinetic/pharmacodynamic
PRB	Proportion of resistant bacteria
PVDF	Polyvinylidene fluoride
RMS	Resistant mutant subpopulations
SCTK	Static concentration time-kill
TOB	Tobramycin
$T_{>MIC}$	Time the concentration exceeds the minimum inhibitory concentration
WGS	Whole genome sequencing
WHO	World Health Organization
WT	Wild type

Chapter 1

Introduction

1.0 Introduction

1.1 Overview

The discovery of antimicrobial agents is one of the cornerstones of successful interventions in the history of medicine. The antibiotic era has allowed the control of infectious diseases and saved countless lives. This resulted in a growing overconfidence that this would be the end of deadly infections caused by bacterial pathogens. However, antimicrobial resistant 'superbugs' have been rapidly emerging, imposing a significant global threat to human health (1-3) as one of the leading causes of death (4). For years antibiotics have been over-prescribed and used inappropriately, leading to the decline in their effectiveness against bacterial pathogens (1, 4). Antibiotic development has slowed substantially due to economic and regulatory disincentives (1). Without effective antibiotics, common infections with these bacterial 'superbugs' will result in greatly increased morbidity and mortality rates (2). Antibiotic monotherapy has been effective in the past against infections caused by susceptible pathogens, but currently monotherapy is often no longer viable in cases of severe bacterial infections (5). Consequently, effective combinations of available antibiotics have been sought and present a highly promising, timely and tangible option to combat antimicrobial resistance.

Cystic fibrosis (CF) patients are especially prone to bacterial infections due to the characteristic overproduction of mucus within their lungs that creates a favorable growth environment (6). Owing to the frequent exacerbations from bacterial respiratory infections, CF patients are often exposed to long and repeated antimicrobial therapies (7). These respiratory infections are due to a range of different pathogens. *Pseudomonas aeruginosa*, the main focus of this thesis, has been found to be the main cause of progressive damage to the lungs of CF patients, resulting in high rates of mortality (8-10). In recent years, some *P. aeruginosa* strains have been found to exhibit

hypermutator phenotype (*i.e.* an increased spontaneous mutation rate) in chronic respiratory infections of CF patients (11-14). This increased mutation rate, paired with the short bacterial generation time, allows hypermutable strains to rapidly adapt to a variety of stressful environments, such as antibiotic exposure (12, 15). Thereby, these strains can gain or enhance their resistance mechanisms (11, 12, 14, 16), making infections by hypermutable *P. aeruginosa* more difficult to treat.

This chapter provides a general background on the antibiotic resistance crisis and the challenges of *P. aeruginosa* respiratory infections in CF patients, including by strains with the hypermutator phenotype. Also included is a summary of the commonly used antipseudomonal antibiotics and the antibiotic dosing strategies currently employed. The pharmacokinetic/pharmacodynamic (PK/PD) principles and considerations for antibiotic combination therapy, including synergy, will be described.

1.2 Antibacterial resistance crisis

The discovery of the first antibiotic penicillin by Alexander Fleming in 1928 underpinned the beginning of the antibiotic era (17). This had a profound impact on human health with rapid and effective treatment options available for previously fatal bacterial infections. This successful use of antibiotics led to the misconception that the age of bacterial infections was coming to an end (18). More than 20 new classes of antibiotics were discovered between the 1930s and 1960s. Since the 1960s there have been limited discoveries of new antibiotic classes. Antibiotic development has plateaued with declining research and development into new antibiotic discoveries (18, 19). This has led to a substantial reduction in the number of newly approved antibiotics concurrent with increasing resistance emergence (**Figure 1**) (20). Notably, in 1940

prior to the therapeutic use of penicillin, the first antibiotic inactivating enzyme penicillinase was identified (21).

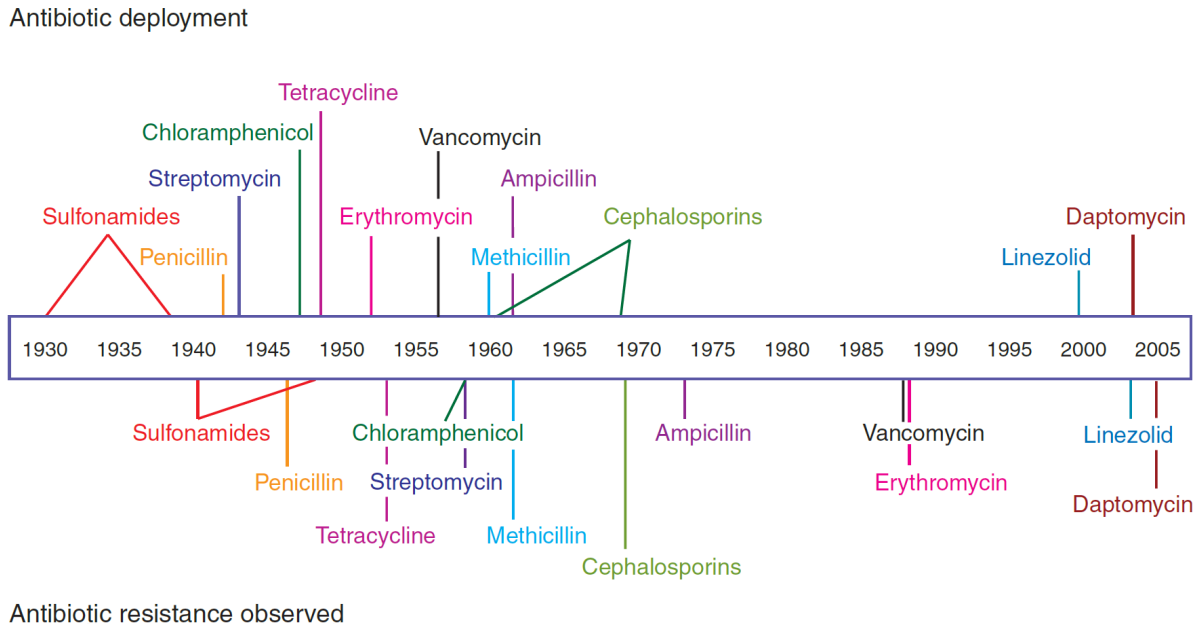

Figure 1: The timeline of approved antibiotics and antibiotic resistance. Above the timeline depicts the year each antibiotic was deployed and below the timeline depicts the year that resistance was observed to each antibiotic. Reprinted with permission from Macmillan Publishers Ltd: Nature Chemical Biology (20), copyright (2007).

Antimicrobial resistance is one of the major global threats to human health according to the World Health Organization (WHO) and the Australian Bureau of Statistics (22-25). Antibiotic-resistant bacterial infections are problematic as they are a major cause of morbidity and mortality worldwide (2). Antibiotic resistance has arisen as a complex and interlinking problem at a societal level from the use of antibiotics in humans and in agriculture, and from environmental pollution (26). Resistance is a natural evolutionary response to antibiotic exposure based on Darwinian notions of selection and survival (27). Recently, it was estimated that 700,000 people worldwide die annually (~80 people every hour) due to antimicrobial-resistant infections; this number has been projected to be 10 million by the year 2050 (28). Currently, humanity faces a dire threat of a return to the pre-antibiotic era.

In response to this threat the WHO first released a global strategy for the containment of this antimicrobial resistance crisis in 2001 (23). Following this example, the Australian Government released a four year National Antimicrobial Resistance Strategy in 2015 (29) and assembled the Antimicrobial Use and Resistance in Australia Surveillance System in 2016 (30). This strategy is currently being implemented by governments, health care professionals, veterinarians, farmers and communities (31). The main goal of this strategy is to minimise the resistance to antimicrobials to allow the continued availability of effective antibiotics for first class human health services (29, 31). Ultimately, this will allow better control of bacterial infections, such as those experienced by CF patients.

1.3 Cystic fibrosis

CF is a life-limiting, autosomal-recessive, hereditary disorder that is predominant in Caucasian populations. Approximately 1 in 2500 newborns (32) and about 100,000 globally are affected by this condition (33). This multisystem disorder affects the lungs, gastrointestinal tract, pancreas, liver and other organs (34) *via* mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The CFTR creates the adenosine triphosphate (ATP)-binding cassette (ABC) transporter ion channel in the plasma membrane that primarily functions as a chloride (Cl^-) ion channel. The CFTR also co-localises and regulates with the epithelial sodium (Na^+) channel (ENaC). There are over 2000 mutations in the CFTR gene that have been identified, with 127 confirmed to cause CF (35). The loss or inactivation of the CFTR gene results in an inadequate amount of CFTR protein being produced resulting in a reduced CFTR function. With this reduced function the mucus glands along with a defective mucociliary clearance (*i.e.* self-clearing mechanism of the bronchi) allow thick, sticky mucus to accumulate. Thus, the airways in the lungs of people with CF become blocked (8). This mucus produces a favorable

environment for bacteria within the respiratory tract (7). Consequently, this often allows chronic respiratory infections to be established which are still the main driver of mortality (8). The main pathogens that infect the CF airways include *P. aeruginosa, Staphylococcus aureus, Haemophilus influenzae* and *Burkholderia cepacia* (6). However, infections by *P. aeruginosa* have been associated with the highest rates of mortality (8-10).

1.3.1 *P. aeruginosa* prevalence and impact in respiratory disease

By age 3 years 97.5% of children with CF have a *P. aeruginosa* infection (36), and between 70 – 80% of CF patients are chronically infected by their teen years (8). This results in permanent lung damage leading to pulmonary insufficiency that causes respiratory failure and eventual mortality (8). Chronic infections occur through genotypic and phenotypic changes in the bacteria. These include increased antibiotic resistance, decreased metabolism, reduced growth rate, lack of motility, deficient quorum sensing and alginate overproduction (37-40). The failure of microbial clearance and creation of a toxic pro-inflammatory local microenvironment within the airways produces a chronic inflammatory response (6). A healthy immune system has phagocytic cells that ingest *P. aeruginosa*, however this mechanism is less efficient in people with CF and *P. aeruginosa* are quite effective at evading the host's immune responses (41). A common trait of people with CF is the need for lengthy antibiotic therapy and life-long exposure to antibiotics, subsequently this often results in the emergence of resistance (42). This is heightened for infections due to *P. aeruginosa*, as it has an exceptional potential to become resistant during antibiotic therapy (43-45) and can also evolve into hypermutators.

1.4 Hypermutable *P. aeruginosa*

Some strains of *P. aeruginosa* have the ability to rapidly increase their mutation rate in a phenomenon known as hypermutation, thereby developing a hypermutator phenotype (12, 46, 47). Hypermutators in natural populations were first identified in a study of *P. aeruginosa* isolates from chronic respiratory infections in CF patients (12). Since their identification, hypermutable *P. aeruginosa* strains have frequently been isolated from CF patients (11-14). Overall, ~27% of isolates obtained from CF patients have been found to be hypermutators and ~45% of patients have been reported to harbour them (**Table 1**). Later, they were also found to be prevalent in chronic respiratory infections of other patients, including those with bronchiectasis or chronic obstructive pulmonary disease (14, 48). Contrastingly, it has been found that the prevalence of hypermutable strains is low in acute respiratory infections by *P. aeruginosa* in non-CF patients (12, 49). In 2000, it was proposed that the signature progressive lung deterioration seen in chronic infections of CF patients favoured adaptation of *P. aeruginosa*, as observed with hypermutators (12). Over a decade ago it was further suggested by Gutierrez and colleagues (49) that the co-selection of hypermutators with adaptive mutations allowing persistence in chronic respiratory infections does not occur in acute infections, hence the lack of hypermutation. The high frequency of hypermutators in chronic infections may also be due to the number of mutations in a range of different genes that allow hypermutation in *P. aeruginosa*, thus are more likely to occur than one specific mutation in a gene as the underlying cause.

The investigation into the prevalence of hypermutators in CF patients in Australia has been limited. As part of an international study, Kenna and colleagues (50) evaluated the emergence of hypermutators in three *P. aeruginosa* populations; clinical isolates from early stages of infection,

Table 1: Prevalence and description of *P. aeruginosa* hypermutators isolated from the lungs of cystic fibrosis patients. Table updated from Oliver 2010 (11).

Year of publication	Isolates/CF patients studied Prevalence of hypermutators	Description
2000 **(12)**	128 isolates/30 patients 20% of isolates/37% of patients	Longitudinal study in chronically colonised CF patients from Spain (1993–1998)
2003 **(51)**	26 isolates/4 patients 15% of isolates/25% of patients	Chronically colonised CF patients from Germany (1985-1992)
2004 **(52)**	35 isolates/21 patients 29% of isolates/24% of patients	Chronically colonised CF patients from Spain (2003)
2005 **(53)**	79 sputum samples/79 patients 54% of patients	Cross-sectional study in chronically colonised CF patients from Denmark (2002–2003)
	141 isolates/11 patients 30% of isolates/73% of patients	Longitudinal study in chronically colonised CF patients from Denmark (1973–1999)
2006 **(54)**	100 sputum samples/100 patients 15% of samples/patients	Cross-sectional study in chronically colonised CF patients from Germany
2007 **(55)**	111 varients from 3 isolates/3 patients	Hypermutator strains accumulated during the observation period of 3-6 years (dominance later in the disease)
2007 **(46)**	12 MDR isolates/11 patients 92% of isolates	Characterisation of 12 genetically distinct MDR CF strains from a multicentre study in Germany (2004)
2007 **(50)**	100 isolates/95 patients 10% of isolates	CF patients with early colonisation from the UK and Belgium
	15 epidemic isolates 13% of isolates	Representatives of CF epidemic strains from the UK, Australia, Germany and Ireland
2007 **(56)**	39 isolates/10 patients 18% of isolates/60% of patients	Longitudinal study in chronically colonised CF patients from Germany (up to 15 years follow-up)
2008 **(57)**	90 isolates/29 patients 17% of isolates/31% of patients	Longitudinal study in chronically colonised CF patients from the USA and Canada (up to 20 years follow-up)
2008 **(47)**	153 isolates/40 patients 31% of isolates/40% of patients	Chronically colonised CF patients from the UK
2009 (16)	136 isolates/36 patients 27% of isolates/50% of patients	Chronically colonised CF patients from France (2001–2003)
2009 **(58)**	11 isolates/3 patients 45% of isolates/100% of patients	Sequential isogenic isolates from CF patients in Germany (over a period of 3-5 years, 1998-2003)
2010 **(59)**	70 isolates/10 patients 54% of isolates/100% of patients	Chronically colonised CF patients from Denmark
2010 **(60)**	38 isolates/26 patients 42% of isolates/46% of patients	Chronically colonised CF patients from Argentina
2011 **(61)**	49 isolates/16 patients 24% of isolates/38% of patients	Longitudinal study from chronically colonised CF patients in France (over 3 years)
2012 **(62)**	6 isolates/3 patients 33% of isolates/33% of patients	Paired longitudinally sampled isolates from chronically colonised CF patients from the UK,
2012 **(63)**	21 isolates/1 patients	Multilocus sequence typing study on isolates obtained from 1 chronically colonised patient from Spain
2013 **(64)**	705 isolates/149 patients 28% of isolates/66% of patients	Chronically infected CF patients from two cities in Brazil
2013 **(65)**	55 isolates/21 patients 20% of isolates/48% of patients	Chronically colonised CF patients from Denmark
2014 **(66)**	27 isolates/2 patients	Longitudinal study in chronically colonised CF patients from Argentina and Denmark (up to 6 and 20 years)
2015 **(67)**	96 isolates/73 patients 30% of patients	Chronically colonised CF patients from Israel (2010-2011)
2017 **(68)**	48 isolates 38% of isolates	Isolates before and after a 2-week course of suppressive therapy
2017 **(69)**	11 isolates/1 patients 64% of isolates	*P. aeruginosa* AUST-02 strain sub-type M3L7 isolates from one CF patient (over 3 months), hypermutable isolates were defined as having *mutL* inactivation
2017 **(70)**	29 isolates/17 patients 31% of isolates/35% of patients	Longitudinal study in chronically colonised CF patients from Australia and Spain over 18-years (1995–2012)
2017 **(71)**	79 isolates/75 patients 15% of isolates	Multicentre study of isolates obtained from CF patients in Spain (2003-2004)

environmental isolates that are considered the source of infection and epidemic, highly transmissible strains. This group utilised four isolates from CF patients at a clinic in Australia, however only phenotypic determination of mutation frequency was carried out on these isolates and there was no description of their individual results. More recently, Sherrard and colleagues (69) used whole genome sequencing to investigate *P. aeruginosa* AUST-02 strain sub-type, M3L7, isolates obtained from one CF patient from Australia over 3 months. This study found an inactivating mutation in *mutL* that was concluded to result in hypermutator phenotype despite no rifampicin mutation frequencies being performed to confirm hypermutation. López-Causapé and colleagues (70) utilised whole genome sequencing to investigate a widespread clone, CC274, determining the phylogeny, interpatient dissemination, and hypermutator and resistance genotypes. This study included eight isolates from different geographical locations around Australia collected in 2007 and 2008, of which 3 were hypermutators. However, the focus of that study was the CC274 clone, and the small number of isolates from Australia collected approximately a decade ago, does not allow determination of the prevalence of hypermutators in one clinical site in circumstances more reflective of contemporary antibiotic prescribing. Therefore, the current state of hypermutator prevalence in CF patients in Australia has not been established; this is one aspect of the studies described in Chapter 5.

Hypermutation occurs through alterations within DNA repair pathways, especially the mismatch repair (MMR) system (72, 73). Proteins involved in these repair pathways assist in the replacement of incorrect nucleotides in replicated DNA and prevent recombination of DNA sequences that are not identical (72). Hypermutation in *P. aeruginosa* often results from a non-functional *mutS* gene (14, 74). Other genes involved include those encoding β-clamp proteins (*mutL* and *mutU*) (75, 76), the basal excision repair genes (*mutY, mutM* and *mutT*) that are

involved in the 7,8-dihydro-8-oxo-deoxyguanosine (8-oxodG or GO) system (72, 77), and the proofreading gene *mutD* (72, 78). Alterations in genes involved in preventing oxidative damage caused by reactive oxygen species, such as the tRNA genes *radA* and *pfpI* in *P. aeruginosa*, can also result in hypermutation (79, 80).

Most of the above DNA repair pathways, especially the MMR system, have frequently been identified to be responsible for hypermutation of *P. aeruginosa* in CF patients (56, 57, 59, 60). Mutations in these genes afford such hypermutators an up to 1000-fold increased spontaneous mutation rate (12, 13, 81) allowing a more rapid development of *de novo* mutational resistance to antibiotics compared to non-hypermutable strains (82). The short generation times of bacteria allow rapid adaptation to stressful and varying environments, which is enhanced by hypermutation (15). The long-term exposure to antibiotic therapies and the lung deterioration seen in CF patients allows these hypermutators an advantage to adapt to the CF lung environment, with their increased mutation rates catalysing the acquisition of adaptive mutations (depicted in **Figure 2**) (13, 15). The spontaneous mutations may arise in a number of regulatory genes including those involved in antibiotic resistance (mechanisms of resistance discussed in Section 1.5). Hypermutators from CF patients often show antibiotic resistance (12, 14) rendering *P. aeruginosa* infections by hypermutable strains particularly difficult to treat (11, 16). The molecular characterisation of hypermutators from an Australian CF clinic is an important component of the studies described in Chapter 5.

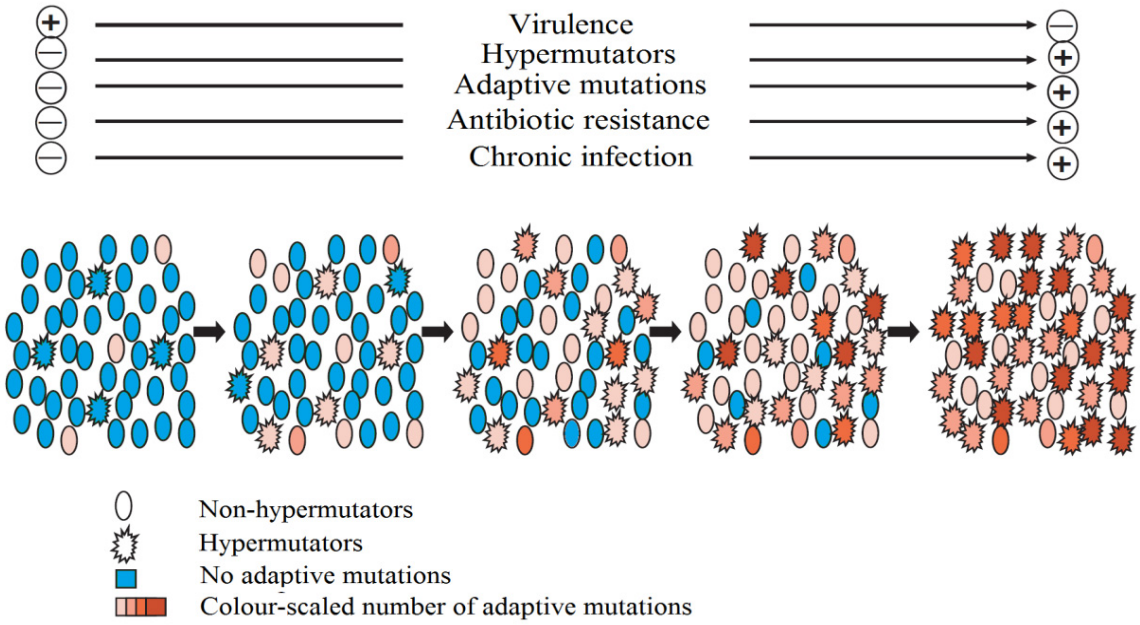

Figure 2: Schematic representation of the role of hypermutators as catalysers on adaptive evolution of *P. aeruginosa* in chronic lung infection of cystic fibrosis patients. Figure adapted from (83).

1.5 Antipseudomonal agents and resistance

P. aeruginosa is capable of surviving high doses of different antibiotics for prolonged periods of time (74) and is a member of the ESKAPE family of pathogens (*Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter species*) (84). As the name suggests, *P. aeruginosa* and these other pathogens can 'escape' the bactericidal effects of antibiotics *via* a wide range of antimicrobial resistance mechanisms. Antibiotic resistance in *P. aeruginosa* is a growing problem worldwide with increased isolation of multidrug-, extensively drug- and pandrug-resistant strains (45). Multidrug-resistance involves resistance to three or more antimicrobial classes, extensive drug-resistance involves resistance to all but one or two antimicrobial classes, and pandrug-resistance involves resistance to all antimicrobial classes (85). The antimicrobial agents (administered intravenously) often used for

the treatment of CF (86-91), which are the focus of this thesis, include the β-lactam, fluoroquinolone and aminoglycoside antipseudomonal antibiotics.

P. aeruginosa shows intrinsic resistance against many antibiotics, with the most important resistance mechanisms including the overexpression of Mex efflux pumps (92-94), antibiotic target site modifications (95), enzymatic inactivation or alteration of antibiotics (96), cell membrane permeability changes that lead to reduced intracellular drug accumulation (94), and biofilm formation (discussed in Section 1.6.4) (97-99). The genes associated with antibiotic resistance may be carried on the bacterial chromosome, plasmids or transposons (100, 101). Common mechanisms of resistance of *P. aeruginosa* are illustrated in **Figure 3**. An understanding of the mechanisms of activity and resistance would allow us to be better equipped to combat these pathogens. These aspects are reviewed below for the antipseudomonal antibiotics relevant in this thesis.

1.5.1 Aminoglycosides

Aminoglycosides bind to the 30S ribosomal subunit of *P. aeruginosa* and thereby affect protein biosynthesis. This occurs *via* inaccurate mRNA translation and disruption to peptide elongation (102). The aminoglycosides tobramycin and gentamicin have also been found to disrupt the bacterial outer membrane (103). Throughout the 1970s and 1980s, aminoglycosides were widely used although oto- and nephrotoxicity was an issue (104). The antibacterial effects of aminoglycosides have been shown to be dependent on the concentration, with more rapid and extensive bacterial killing observed with higher concentrations (105-107). Aminoglycosides have also been found to exhibit a delay in bacterial regrowth with the observation of cellular recovery after exposure, known as a postantibiotic effect (PAE) (108, 109). The PAE duration has been found to be related to the antibiotic concentration (110, 111), the duration of antibiotic exposure

(112) and the inoculum size (112, 113). It has been proposed in the past that a PAE may suppress susceptible populations from regrowing (114).

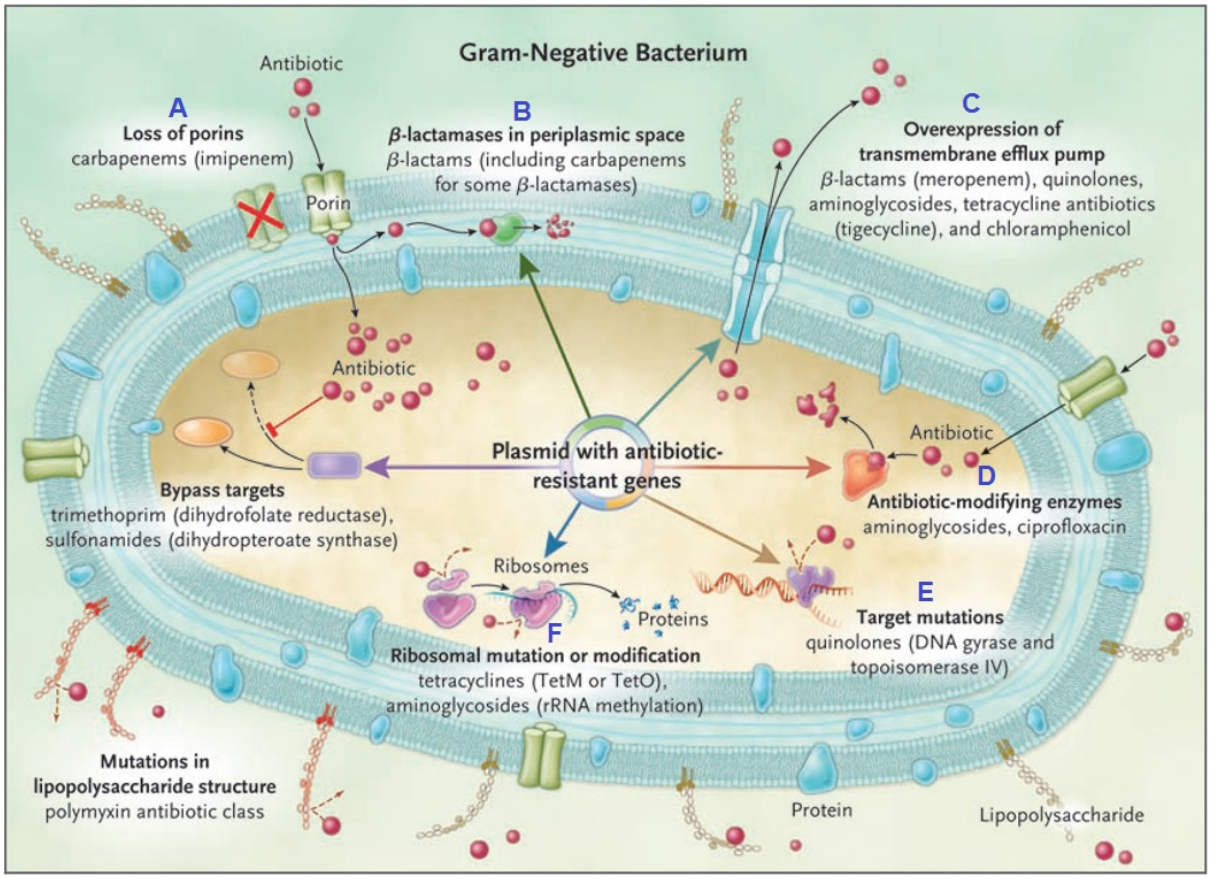

Figure 3: Resistance mechanisms of Gram-negative pathogens, including *P. aeruginosa*. Several important mechanisms of resistance exist in *P. aeruginosa* for aminoglycosides, β-lactams and fluoroquinolones: including the loss of porins (**A**); the presence of β-lactamases in the periplasmic space (**B**); increased expression of the transmembrane efflux pump (**C**); the presence of antibiotic-modifying enzymes (**D**); target site mutations (**E**); and ribosomal mutations or modifications (**F**). Red spheres indicate antibiotics. Reproduced with permission from Peleg and colleagues (115), Copyright Massachusetts Medical Society.

Aminoglycoside resistance in *P. aeruginosa* commonly occurs *via* the up-regulation of the MexXY-OprM efflux pump (101, 116-118). The MexXY-OprM efflux pump is induced dependent on drug-ribosome interactions (119). The up-regulation of this efflux pump frequently produces adaptive resistance from the overexpression of the MexY component, mutations in the

local repressor gene of the mexXY operon (*mexZ*) or *de novo* mutations during treatment (116, 119, 120). Aminoglycosides used in monotherapy against *P. aeruginosa* infections often fail due to this selection of resistant mutants (44, 82, 121). Additional common mechanisms of aminoglycoside resistance in *P. aeruginosa* include enzymatic inactivation or decreased permeability of the membrane (122). The enzymatic inactivation of aminoglycosides intracellularly occurs *via* phosphoryltransferases that phosphorylate, acetyltransferases that acetylate and nucleotidyltransferases (also known as adenyltransferases) that adenylate aminoglycosides (122-125). Membrane permeability is altered when mutations arise in essential outer membrane porins (proteins that form channels) that are required for the hydrophilic outer membrane barrier of *P. aeruginosa* to perform effectively (126, 127).

1.5.2 Beta-lactam antibiotics

There are four classes of β-lactam antibiotics, carbapenems, cephalosporins, penicillins and monobactams, that all act on *P. aeruginosa* by inhibiting cell wall synthesis (128). The carbapenems include imipenem and meropenem (129). The cephalosporins are divided into 1st, 2nd, 3rd, 4th and 5th generations. Currently, the third generation cephalosporins are among the most widely used subclass of antibiotics and include ceftazidime (130). The penicillins include piperacillin (131), while the monobactam class only has one approved antibiotic, which is aztreonam (132). β-Lactam antibiotics inhibit synthesis of the cell wall by binding to specific penicillin-binding proteins (PBP) anchored to the bacterial cytosolic membrane (133, 134). Unlike aminoglycosides, the β-lactams exhibit time-dependent killing in both *in vitro* and animal studies (105-107). β-Lactams also show a saturation of the killing rate (106, 135). Thus, increases in concentration often have minimal effect on the rate of killing and β-lactams require longer antibiotic exposure for antibacterial effects to be exhibited (136). The WHO has described

carbapenem-resistant *P. aeruginosa* as one of the priority 1 critical pathogens that requires new antimicrobial agents, thus posing a great threat to human health and necessitating urgent action (137).

The common resistance mechanisms for β-lactam antibiotics include active efflux *via* the MexAB-OprM and MexCD-OprJ efflux pumps (75, 117, 138), modification of the PBP target sites, enzymatic inactivation by β-lactamases (75, 138), and changes to the porin type or expression levels (or loss) of outer membrane porins. The loss of the porin OprD is the most prevalent mutation that confers resistance to imipenem and reduced susceptibility to meropenem (139). All *P. aeruginosa* have an inducible chromosomal AmpC β-lactamase that irreversibly modifies and inactivates some β-lactams (75, 138). The hyperproduction of AmpC can occur when the negative regulator *ampD* is inactivated (140). Resistance to ceftazidime and aztreonam often occurs with the overexpression of AmpC (141). Resistance to the β-lactams meropenem and ceftazidime is related to β-lactamase activity but not always through individual mutations (142). Many random mutations have subsequently allowed an increased extended spectrum of resistance for some β-lactamases (143). β-Lactamase inhibitors, such as avibactam, clavulanic acid, sulbactam, and tazobactam, are utilised to inactivate the β-lactamase enzyme and allow the β-lactam to become effective once again (144, 145).

1.5.3 Fluoroquinolones

Fluoroquinolones were introduced in 1987 with the aim of preventing DNA replication *via* inhibiting bacterial type II topoisomerases (146), including DNA gyrase (DNA topoisomerase II) and DNA topoisomerase IV (147). These type II topoisomerases act on DNA replication to relax supercoiled DNA, unwinding over-twisted DNA (147-149). The use of a low fluoroquinolone concentration results in the impairment of DNA replication whilst higher concentrations lead to

cell death (148, 149). Fluoroquinolones, similar to aminoglycosides, are classed as concentration-dependent antibiotics, with higher concentrations associated with more rapid bacterial killing (105-107).

Fluoroquinolone resistance in *P. aeruginosa* often results from efflux pump overexpression and target site mutations, with amino acid substitutions altering the target protein structures and lowering the binding affinity (150, 151). Increased minimum inhibitory concentrations (MIC) correlate with increasing numbers of mutations within these subunits (152, 153). A majority of these mutations occur within defined regions of the subunits, designated the quinolone resistance-determining regions (154). High-level ciprofloxacin resistance and cross-resistance to β-lactams have been observed following exposure to ciprofloxacin concentrations below the MIC (155). Furthermore, target site mutations can be enhanced by further mutations that result in the overexpression of efflux pumps (156, 157).

Efflux pump inhibitors, such as phenylalanine-arginine-β-naphthylamide (PAβN) (158), inhibit this efflux-mediated mechanism of resistance (92, 159-161). The combinations of antibiotics with inhibitors have been found to be promising (162-164). The combination PAβN with ciprofloxacin, which is heavily effluxed by most Mex efflux pumps (117) is used to assess efflux-mediated ciprofloxacin resistance in Chapter 2.

1.5.4 The impact of hypermutation on the susceptibility to antimicrobial agents

Antibiotic resistance is a major concern for CF patients, and *P. aeruginosa* hypermutators have an exceptional potential to enhance their resistance. Along with the first identification of hypermutators by Oliver and colleagues (12), the characteristic increased antibiotic resistance to diverse antimicrobials was observed with this phenotype. Following on from this, the same group constructed a *mutS* knockout strain, PAOΔ*mutS*, from the PAO1 wild-type strain, to

investigate this hypermutator phenotype (52). These strains differed only by the absence of *mutS*, which was evident through the MIC values for the predominant population of the isogenic strains being near identical. Interestingly, the hypermutable PAOΔ*mutS* strain produced colonies above the MIC for the predominant population. These were labelled accordingly as resistant mutant subpopulations (RMS). Subsequent studies by the same investigators using these strains have continued to observe comparable MICs and the presence of RMS (165, 166). Another group investigated hypermutator phenotypes *via* the construction of *mutY*, *mutT* and *mutM* knockout strains from the PAO1 strain (167). These hypermutable strains produced comparable MICs to their parental PAO1 strain, alike the aforementioned studies with PAOΔ*mutS*. RMS were again present for the hypermutable strains, with the greatest numbers observed for PAOΔ*mutT*, followed by PAOΔ*mutY* and then PAOΔ*mutM*.

The hypermutator phenotype was further assessed for antibiotic resistance in isolates from the lungs of CF patients and resistance was found to be substantially greater for hypermutators compared to non-hypermutators (16, 53, 60). One of these studies also reported the frequent observation of RMS for hypermutators (60). These RMS observed in CF isolates often have greater resistance than the predominant bacterial population (64). Therefore, even when the MICs for hypermutators are not found to be elevated, the RMS will likely arise in the presence of antibiotics. This needs to be considered when treating infections involving these hypermutable *P. aeruginosa* (this will be discussed further in Section 1.6.4).

1.6 Approaches to antimicrobial treatment

The development of effective antimicrobial treatment strategies is a necessity to eradicate pathogens at the site of infection (168). The pharmacology of antimicrobial therapy entails two distinct components, pharmacokinetics (PK) and pharmacodynamics (PD) (106, 169). PK refers

to the time-course of the drug concentration in different fluids, *e.g.* serum or epithelial lining fluid. When a drug is administered into the human body it undergoes absorption, distribution, metabolism and excretion. These are characterised by primary PK parameters (total body clearance, volume of distribution) and secondary parameters (half-life, bioavailability) that directly impact the time-course of the drug. PD refers to the drug exhibiting an effect at the target site *via* its mechanism of action (170). PK/PD integrates the two components to describe the relationship between the time-course of drug concentration and that of the effect elicited.

Susceptibility information obtained through the use of MIC frequently assists clinicians in the choice of dosage regimens. However, MIC values can often be misleading. This is due to the total cell counts being too low to allow the resistant subpopulations to manifest over the time of the test (171, 172). In the case of broth microdilution (the reference method), there are generally only ~5x 10^5 CFU per well, and for agar dilution only ~10^4 CFU per spot (173). *P. aeruginosa* frequently harbours resistant subpopulations at higher inocula (171, 172, 174, 175). Furthermore, MIC values are also limited to the detail provided by summary endpoints. In other words, the MIC exhibits an "all or none" (growth *versus* no growth, killing *versus* no killing; as crudely assessed by 'turbid' *versus* 'not turbid' media in an MIC test) phenomena at a single time-point (176). Thus, the time-course of the individual PK and PD processes, including the changing antibiotic concentrations that are observed in the human body, is not enlisted (168, 177).

Notwithstanding the limitations of MICs, they have proven very useful as an *in vitro* measure of PD activity of an antibiotic against a given strain when incorporated with PK measures of exposure to the antibiotic. The choice of drug, dose of drug, and schedule of administration are vital in successful outcomes for antimicrobial therapy; careful consideration of these factors

would increase the chance of clinical success (104, 178). As a result antibiotic dosage regimens are often based on the correlation between the magnitude of their effect and PK/PD indices (179).

1.6.1 Pharmacokinetic/pharmacodynamic indices

The PK/PD indices support antibiotic dose selection and have been found to be capable of improving clinical outcomes (105, 106, 169, 180-183). However, in some cases, PK/PD targets are not based on the suppression of antibiotic resistance (180, 182, 184). The PK/PD index is based on the ratio of a measure of *in vivo* exposure to the antibiotic under consideration and susceptibility of a bacterial strain to that agent (MIC) (106). There are three commonly used PK/PD indices: the area under the unbound (free) drug concentration-time curve divided by the MIC (*f*AUC/MIC), the maximal unbound drug concentration divided by the MIC (fC_{max}/MIC), and the time that the unbound drug concentration exceeds the MIC over 24 h ($fT_{>MIC}$) (178) (**Figure 4**).

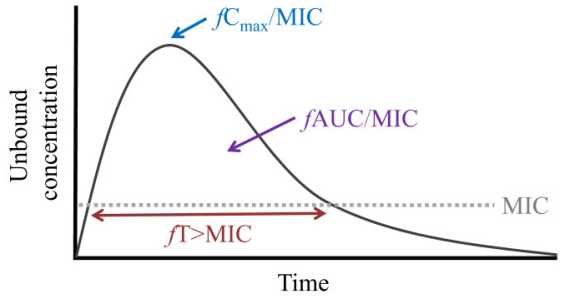

Figure 4: The pharmacokinetic/pharmacodynamic (PK/PD) indices associated with antibiotic efficacy: the area under the free antibiotic concentration-time curve divided by the MIC (*f*AUC/MIC), the maximal free antibiotic concentration divided by the MIC (fC_{max}/MIC), and the time that the free antibiotic concentration is above the MIC ($fT_{>MIC}$).

In vitro and *in vivo* studies are utilised to establish the most predictive PK/PD index and the targets for various magnitudes of bacterial killing (169). The most important PK/PD index for aminoglycosides and fluoroquinolones was suggested to be the *f*AUC/MIC as it has been found

to be the best predictor for bacterial killing (106). However, the fC_{max}/MIC which is based on a single time-point concentration within a dosage interval has also been considered to be a suitable predictor for aminoglycosides and fluoroquinolones (106, 169, 185). In contrast, $fT_{>MIC}$ is considered the best predictive index for therapeutic success of β-lactam antibiotics (106).

It is important to note that the fAUC, fC_{max} and $fT_{>MIC}$ and corresponding values for total drug concentration (AUC, C_{max} and $T_{>MIC}$) will change depending on dose and frequency when the clearance and half-life are constant. With a given daily dose, the administration schedule has no impact on the fAUC, as it remains constant regardless of the shape of the concentration-time curve. However, the shape of the concentration-time curve has been found to have an impact on some antibiotics in the past (186, 187). This is represented in **Figure 5**. The left panel shows that a fourfold-higher dose increases both C_{max}/MIC and AUC/MIC, with a longer $T_{>MIC}$. To differentiate among the three PK/PD indices, dose-ranging dose-fractionation studies are required (**Figure 5**, right panel). A lower C_{max}/MIC and longer $T_{>MIC}$ dose can be achieved with using a dose administered every 2 h compared to a fourfold-higher dose administered every 8 h. As stated above, with the same daily dose the AUC/MIC of the two regimens over 24 h is identical.

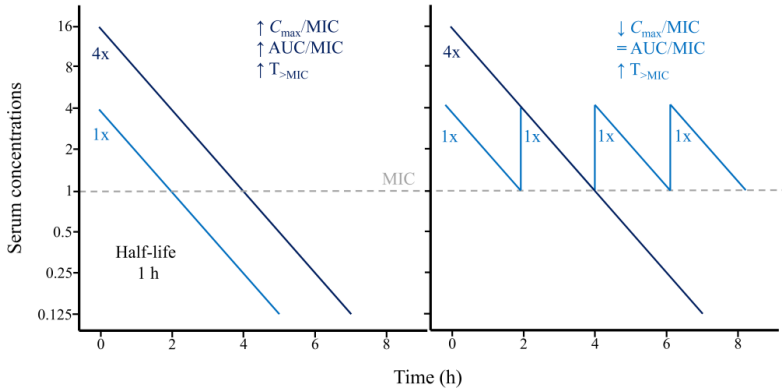

Figure 5: The effect of increasing the dose or changing the dosing interval of an antibiotic on the three pharmacokinetic/pharmacodynamic (PK/PD) indices: AUC/MIC, C_{max}/MIC and $T_{>MIC}$. Figure adapted from Craig (135), with permission.

The *f*AUC/MIC relies on a specific time-averaged exposure to a particular antibiotic and suggests the same extent of bacterial killing regardless of the shape of the concentration-time curve. As both aminoglycosides and fluoroquinolones are described to be well represented by both AUC/MIC and C_{max}/MIC it seems apparent that the shape of the concentration-time curve may impact the antibacterial effects; yet it appears that this has not been systematically investigated. Currently used dosage regimens are often based on these traditional PK/PD indices (179). However as highlighted above, there are limitations to these indices that require investigation. Evaluation of the AUC/MIC with regard to the shape of the concentration-time curve for fluoroquinolones and aminoglycosides will be undertaken in Chapters 2 and 3. A more accurate quantitative description of the antibacterial drug effects for different regimens and subpopulations to improve the efficacy of antibiotic treatment can be established through the full time-course of PK and PD. This can be achieved through mechanism-based modelling (MBM).

1.6.2 *In silico* evaluation

The need to evaluate the time-course of PK/PD relationships in antibacterial development has now been recognised by regulatory agencies (178). Nevertheless, currently used older antibiotics have often not been reviewed with these recent techniques. MBM enables researchers to investigate the time-course of antimicrobial PK/PD on bacteria and quantitatively characterise the interactions between antibiotics and bacteria (177, 188-192). The use of MBM benefits researchers in reducing time and monetary burden associated with evaluating dosing strategies experimentally or clinically (190, 193).

Another *in silico* method being utilised as an integral contributor for the investigation of biological systems is bioinformatics, the mathematical analysis of biological data. This method is used in parallel with high-throughput whole genome sequencing for characterisation of pathogen

genomes allowing genes to be decoded such as those related to the mechanisms of antimicrobial resistance (194-198). There is a great need for robust, unequivocal and portable programs for strain genotyping that suggest the identification, links between strain genotype, clinical outcome, antibiotic resistance and epidemiology for *P. aeruginosa* (194). Next-generation sequencing coupled with bioinformatics is the current driver for the advancement of new technologies that incorporate the connection between bacteriology and the clinic (197, 198).

Chapter 5 utilises bioinformatics and whole genome sequencing to characterise *P. aeruginosa* hypermutators from respiratory infections of CF patients. The outcomes of this characterisation support the choice of antimicrobial therapy to be used to combat hypermutators. MBM can be used in parallel with *in vitro* data to aid the improvement of antimicrobial therapy (168, 189, 193, 199-201). Chapter 3 develops MBM to evaluate the time-course of the total and resistant population during aminoglycoside therapy. MBM will also be used in Chapter 6, where the antibacterial effects from a β-lactam in combination with a fluoroquinolone will be examined.

1.6.3 Antimicrobial combination therapy

Monotherapy is no longer viable in cases of some bacterial infections caused by *P. aeruginosa* (5). Due to the rapid rise of resistance to many or all antibiotics in monotherapy, a more promising and timely strategy to overcome this worldwide problem is required. A solution to suppress resistance emergence may be the use of combination therapy (202). Synergy between antimicrobials may increase therapeutic success (203). Two types of synergy between antimicrobials, subpopulation and mechanistic synergy, have been described (189); these are illustrated in **Figure 6**. Subpopulation synergy involves the first antibiotic killing the resistant subpopulation(s) of the second antibiotic, and *vice versa*. Mechanistic synergy involves the

mechanism of action of a second antibiotic enhancing the rate of killing of the first antibiotic (189).

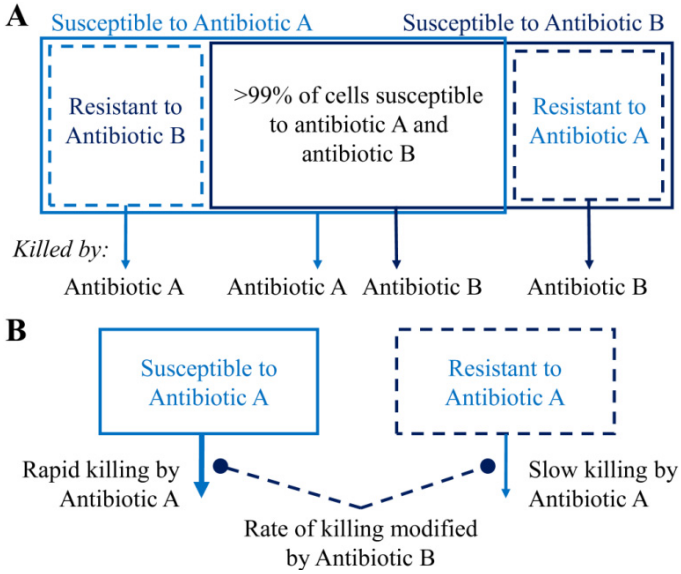

Figure 6: The concepts of synergy. (Panel **A**) Subpopulation synergy. Bacterial subpopulations inside the lighter blue box are susceptible to antibiotic A. Subpopulations inside the darker blue box are susceptible to antibiotic B. The subpopulations in the blue dashed boxes are resistant to either antibiotic A and B. The combination of antibiotic A and B will kill all bacterial subpopulations because there is no subpopulation resistant to both antibiotics. (Panel **B**) Mechanistic synergy. For antibiotics with different mechanisms of action. Antibiotic B enhances the rate of killing by antibiotic A. Figure adapted from Landersdorfer and colleagues (189), with permission.

These mechanisms of synergy are not mutually exclusive as they may both operate simultaneously. These forms of synergy will be discussed further in Chapter 6. Some considerations with combination therapy include the potential risk of increased toxicity and possible drug interactions with other medications (204). With proper consideration of these aspects, combination therapy may prove to be the best strategy against these bacterial 'superbugs.' The next section will provide an overview of studies using aminoglycosides, β-lactams and fluoroquinolones against hypermutators.

1.6.4 Antipseudomonals against hypermutators

As described in Section 1.5.4, simultaneous with the discovery of hypermutable *P. aeruginosa* was the observation of high rates of resistance to antimicrobials for these hypermutators (12, 16, 46, 53). The observation of RMS highlighted that the MIC of hypermutators is not the best indicator for the susceptibility likely to be observed during treatment (52, 60, 64, 165). A quantitative description of the time-course of antibacterial effects on the total and resistant bacterial populations, such as that observed in static concentration time-kill assays, would give more detail to tailor treatment for these hypermutators.

Soon after the first identification of hypermutator *P. aeruginosa* in CF patients (12), Oliver and colleagues (165) demonstrated identical MICs between non-hypermutable PAO1 and hypermutable PAOΔ*mutS* at 16 h, remaining unchanged for PAO1, however by 36 h MICs were elevated for PAOΔ*mutS*. This study further evaluated the antibacterial effects for two antibiotics, imipenem and ciprofloxacin. The authors showed more pronounced antibacterial effects for 4 and 8 mg/L imipenem against a low inoculum of PAO1 (MIC_{36h} of 1 mg/L) than PAOΔ*mutS* (MIC_{36h} of 16 mg/L) in 24-h static concentration time-kill assays (left panel, **Figure 7**). The same study also showed that 0.5 and 1 mg/L ciprofloxacin only suppressed regrowth of PAO1 (MIC_{36h} of 0.06 mg/L), whilst PAOΔ*mutS* (MIC_{36h} of 1 mg/L) exhibited regrowth after 6 h (right panel, **Figure 7**) (165).

This study highlighted the impact of hypermutation on the emergence of resistance for *P. aeruginosa* through an evaluation of the time-course of the total and resistant populations. This study was only carried out with two concentrations over 24 h. A wider range of concentrations and the use of a longer time-period may have resulted in further information regarding the killing kinetics and emergence of resistance. Also this study was carried out at a

low inoculum that likely enabled greater antibacterial effects than would be observed at a higher inoculum representative of a typical respiratory infection in CF patients (165).

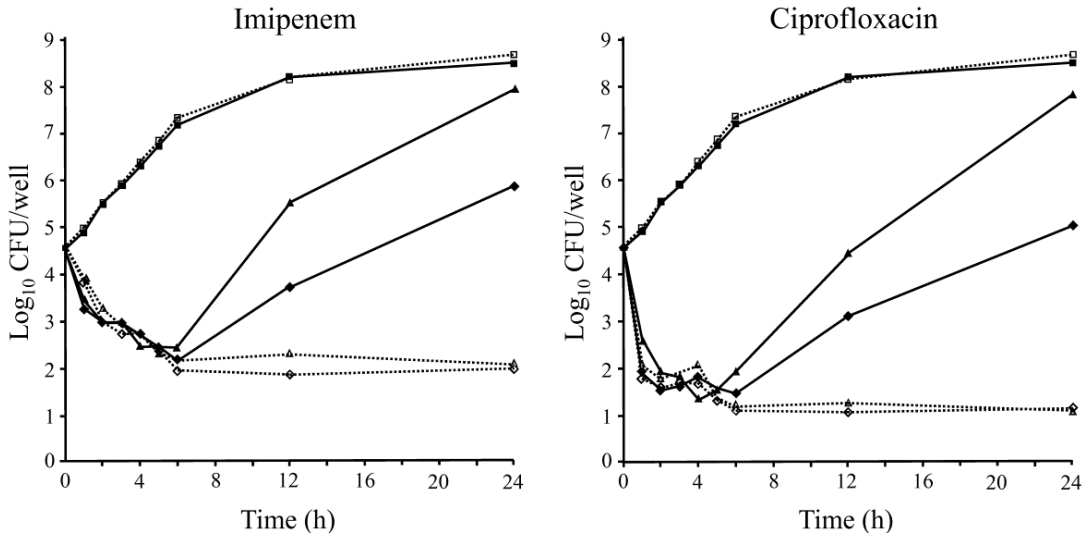

Figure 7: The antibacterial effects in static concentration time-kill studies for *P. aeruginosa* PAO1 (dashed lines and open symbols) and PAOΔ*mutS* (solid lines and solid symbols). Left panel is imipenem killing kinetics. Squares, control with no antibiotic; triangles, imipenem at 4 mg/L; diamonds, imipenem at 8 mg/L. Right panel is ciprofloxacin killing kinetics. Squares, control with no antibiotic; triangles, ciprofloxacin at 0.5 mg/L; diamonds; ciprofloxacin at 1 mg/L. Figure adapted from Oliver and colleagues (165), with permission.

Several years later, Driffield and colleagues (99) suggested that biofilm growth may enhance the rate of mutagenic events, thus heightening the opportunity to derive permanent mutations in the MMR system leading to hypermutator phenotype and the direct selection of mutations that produce antibiotic resistance. They further suggested that hypermutators in biofilm growth would provide a source for antibiotic resistance. The authors compared biofilm growth with planktonic growth of non-hypermutable PAO1 over 72 h. A hypermutable strain was not utilised in these studies, instead the conclusions were based on increases in mutability and gene expression of PAO1 (99). This study led to other studies that used the static biofilm growth model against PAO1 and several PAO1 knockout mutants, *mucA* and *mutS* individual and combination

deficient mutants, to represent mucoid (alginate overproduction) and hypermutator phenotypes, respectively (205, 206).

In a 24-h static biofilm growth model, ceftolozane against PAO1, PAOΔ*mucA*, PAO1Δ*mutS* and PAO1Δ*mucA*Δ*mutS* was shown to always suppress resistant mutants in the biofilm, even with 1× the MIC. Ciprofloxacin and meropenem each required 16× the MIC to largely suppress resistant mutants, whilst ceftazidime at 16× the MIC was not sufficient to suppress resistant mutants (206). This study may have produced more resistant mutants had it been run for longer than 24 h. In a static biofilm growth model over 7 days, azithromycin exhibited bactericidal effects against PAO1, PAOΔ*mucA*, PAO1Δ*mutS* and PAO1Δ*mucA*Δ*mutS* in biofilm growth; however resistant mutants were selected (205). This resulting resistance was examined with hyperexpression of the MexCD-OprJ efflux pump from the knockout of the negative regulator *nfxB* in these strains. This revealed cross resistance to both ciprofloxacin and cefepime as well as hypersusceptibility to imipenem and tobramycin (205). This hypersusceptibility to β-lactams and aminoglycosides was further evaluated by the same group, comparing biofilm with planktonic growth. The results showed that the inactivation of *nfxB* also impaired the MexAB-OprM and MexXY-OprM efflux pumps (207), hence the resulting hypersusceptibility to β-lactams and aminoglycosides.

The above biofilm growth studies exhibited the emergence of antibiotic resistance with hypermutators (205, 206). This was highlighted by another study using a flow-cell biofilm model in which ciprofloxacin 2 mg/L (AUC/MIC of 384) in monotherapy over 4 days was evaluated against PAO1 and PAOΔ*mutS* (208). Ciprofloxacin exposure was found to be insufficient to suppress the emergence of resistance of both hypermutable and non-hypermutable *P. aeruginosa*, despite the ciprofloxacin exposure exceeding the accepted PK/PD target. However, one-step mutations (efflux pump overexpression) were found to be the cause of

resistance for PAO1, whilst two-step mutations (additional GyrA and GyrB target site alterations) were responsible in PAOΔ*mutS* (208). The relationship of hypermutator phenotype with biofilm development and phenotypic diversification was also assessed in a flow-cell biofilm assay (209). MMR system deficient hypermutators had no advantage over PAO1 in planktonic growth. However, for the first time it was demonstrated that MMR system deficient hypermutators had greater tendency towards micro-colony development, phenotypic diversity and morphological colony variants, than PAO1 in biofilm growth (209).

The concentration-time profiles of a common meropenem regimen (1g every 8 hourly for 24 h, with short infusions) was simulated (C_{max} of 56.1 mg/L and $t_{1/2}$ of 0.45 h) in a dynamic *in vitro* one-compartment model. This regimen was not sufficient to suppress the selection of resistant mutants of PAO1 and a clinical hypermutable *P. aeruginosa* isolate (from a wound swab of an Intensive Care Unit patient) (210). This study showed extensive emergence of resistance for the hypermutator by 16 h. PAO1 did not show a similar population of resistant mutants until 24 h. The use of a one-compartment model was intended to represent patients receiving this therapy, however the study was only carried out for 24 h. Using this treatment over a longer time period would have allowed further evaluation of this regimen, also to assess its efficacy with more doses against PAO1. The lack of a growth control (*i.e.* antibiotic-free treatment) also prevented the validation that the effects observed were due to the presence of meropenem. One-compartment models are associated with bacteria being flushed out of the model along with the drug of interest, unlike the hollow fibre infection model that traps bacteria in the extra-capillary space thereby preventing them being washed away. The hollow fibre infection model also uses a closed system that lowers the risk of contamination compared to one-compartment models (211, 212).

The need for combinations of antimicrobials to thwart hypermutable *P. aeruginosa* has been recognised for some time (165). Monotherapy of antibiotics against hypermutators is likely to result in treatment failure; due to the presence of pre-existing RMS and their subsequent ascent (52, 60, 64, 165) (this will be addressed further in Chapter 4). Preliminary synergy studies (comparing both MIC and minimal bactericidal concentration values) have been carried out with different antibiotic classes combined against PAOΔ*mutS* and found to minimise this selection and ascent of resistant mutants to dominance (165).

Macia and colleagues (213) utilised a murine model to investigate the monotherapy of ciprofloxacin 20 mg/kg 6-hourly (*f*AUC/MIC of 385, fC_{max}/MIC of 60) and tobramycin 10 mg/kg 6-hourly (*f*AUC/MIC of 43, fC_{max}/MIC of 19) over 96 h. Both monotherapies were unsuccessful at achieving bacterial killing and had observable resistance emergence against hypermutable PAOΔ*mutS* despite PK/PD target attainment, whilst bacterial killing and resistance suppression occurred against non-hypermutable PAO1. These data suggest that PK/PD targets for antimicrobial monotherapy are not applicable for infections caused by hypermutable strains. However, tobramycin with ciprofloxacin in combination in this murine model resulted in synergistic killing and suppression of resistance (213). The same research group assessed ceftazidime 70 mg/kg 8-hourly ($fT_{>MIC}$ of 54%) over 96 h in mono- and combination therapy with tobramycin or ciprofloxacin (166). Once again *in vivo* resistance suppression was observed for all combinations against PAO1 and hypermutable PAOΔ*mutS*. Due to the inherent ethical difficulties relating to collection of samples at multiple time-points in animals, these murine studies report only three time-points: 24 h before, immediately prior to the first dose, and at the end of treatment. A more detailed systematic evaluation of the time-course can be achieved using *in vitro* models, including the dynamic hollow fibre infection model, to better understand the

bacterial killing kinetics and emergence of resistance. The latter would support the selection of antibiotic combinations against these pathogens.

Current treatment guidelines recommend dual antibiotic therapy to treat pulmonary infections in CF patients (214). However, antibiotic therapies require urgent re-evaluation as hypermutable *P. aeruginosa* strains are prevalent in the lungs of CF patients and are proving to be a therapeutic problem. This thesis incorporates a broad, systematic evaluation of clinically important antibiotics in monotherapy against PAO1 and hypermutable PAOΔ*mutS* (Chapter 4). Furthermore, the combination of fast-acting antibiotics, tobramycin and ciprofloxacin, is comprehensively studied in static *in vitro* studies against PAO1 and hypermutable PAOΔ*mutS* (Chapter 4). Based on the susceptibility of clinical hypermutators (characterised in Chapter 5) and preliminary screening of antibiotic combinations against clinical hypermutators in static concentration time-kill studies, the combination of meropenem and ciprofloxacin is chosen for investigation. The preliminary evaluation of meropenem and ciprofloxacin against PAO1 and hypermutable PAOΔ*mutS* in static *in vitro* models, leads to the systematic evaluation against clinical hypermutators (Chapter 6). This meropenem and ciprofloxacin combination is then evaluated in a hollow fibre infection model study that simulates the antibiotic concentration-time profiles in epithelial lining fluid as would be observed in patients with CF (Chapter 6).

1.7 Summary

Identifying new dosing strategies to combat hypermutable and non-hypermutable *P. aeruginosa* is essential for treating chronic respiratory infections in CF patients. This thesis investigates the steps leading to developing a synergistic approach; proposing that novel antibiotic combination dosing strategies hold excellent promise to combat these 'superbugs' and treat these infections in patients. Antimicrobial therapy against hypermutators needs to be better evaluated to enable the improvement of treatment. The utilisation of static concentration time-kill studies, dynamic *in vitro* systems and MBM-based PK/PD modelling would aid investigation of the antibacterial time-course for antibiotics in combination. Hence, this is expected to result in an increased understanding of how combinatorial antibiotic regimens can be optimised.

1.8 Statement of hypotheses & aims

1.8.1 Hypotheses

The **Hypotheses** that are tested for this project are that:

1. Antibiotic concentration *versus* time profiles with different shapes at the same *f*AUC/MIC can determine the success of bacterial killing and suppression of resistance for fluoroquinolones and aminoglycosides.
2. Hypermutable *P. aeruginosa* are more difficult to treat and emergence of resistance to commonly used antibiotics is elevated, in comparison to non-hypermutable strains.
3. Hypermutable *P. aeruginosa* are prevalent in respiratory infections of CF patients in Australia and exhibit elevated antibiotic resistance.
4. Synergistic combination dosing regimens of available antibiotics, administered with consideration of PK in CF patients, can maximise bacterial killing and suppress resistance emergence in hypermutable *P. aeruginosa* obtained from CF patients.

1.8.2 Project aims

To address these hypotheses, the A**ims** were to:

1. Identify the shape of the concentration *versus* time profile at the same *f*AUC/MIC that maximises bacterial killing and suppresses emergence of resistance in *P. aeruginosa* for a fluoroquinolone and an aminoglycoside.
2. Compare the extent of bacterial killing and suppression of resistance emergence between hypermutable and non-hypermutable *P. aeruginosa*, which can be achieved with clinically important antibiotics.
3. Compare antibiotic susceptibility of recent *P. aeruginosa* isolates from respiratory infections of CF patients; and determine the prevalence of hypermutators and use whole genome sequencing to characterise the cause of hypermutation.
4. Evaluate a promising synergistic antibiotic combination using clinically relevant regimens in a dynamic *in vitro* model to combat hypermutable *P. aeruginosa* of CF patients.

1.9 Thesis layout

The research presented herein takes the following steps to allow the identification of a promising antibiotic combination to suppress the emergence of resistance in a hypermutable *P. aeruginosa* clinical isolate. Chapter 2 of this thesis addresses **Hypothesis 1** *via* **Aim 1** through exploring the antibacterial effects that result from differently shaped concentration *versus* time profiles at the same *f*AUC/MIC for a fluoroquinolone, in 24-h static concentration time-kill experiments. Chapter 3 of this thesis further assesses **Hypothesis 1** *via* **Aim 1** through evaluating the impact of the shape of the aminoglycoside concentration *versus* time curve on the antibacterial effects. This is achieved using static concentration time-kill assays and MBM to assess the time-course of the total and resistant populations. In addition, Chapter 3 begins to explore **Hypothesis 2** *via* **Aim 2** through the inclusion of a hypermutable laboratory strain. Chapter 4 further addresses **Hypothesis 2** in the **Aim 2** studies by systematically evaluating monotherapy of clinically important antipseudomonal antibiotics in 48-h static concentration time-kill experiments. Chapter 4 also broadly assesses a synergistic antibiotic combination of two fast acting antibiotics, tobramycin and ciprofloxacin, against hypermutable and non-hypermutable *P. aeruginosa*. Chapter 5 of this thesis addresses **Aim 3** and **Hypothesis 3** by determining the susceptibility to clinically important antibiotics and the prevalence of hypermutators in recent isolates collected from respiratory infections of CF patients attending an Australian clinic. Subsequently, Chapter 5 utilises whole genome sequencing and bioinformatics to characterise the hypermutable *P. aeruginosa* isolates. The antibiotic susceptibilities of these clinical hypermutators aid the selection of a prospective antibiotic combination to be further investigated. Chapter 6 evaluates **Hypothesis 4** *via* **Aim 4** through 72-h static concentration time-kill assays and studies in the latest dynamic *in vitro* model, with data subjected to MBM, to assess the combination of current meropenem with ciprofloxacin regimens to combat clinical hypermutable *P. aeruginosa* strains.

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Chapter 2

Shape does matter: short high-concentration exposure minimizes resistance emergence for fluoroquinolones in *Pseudomonas aeruginosa*

Preamble

P. aeruginosa is one of the most challenging pathogens, causing infections that frequently result in mortality. This Chapter examines one of the commonly used antipseudomonals, the fluoroquinolone ciprofloxacin. The *f*AUC/MIC has been reported in the literature as one of the most predictive PK/PD targets for bacterial killing by fluoroquinolones. This PK/PD index was examined for ciprofloxacin by evaluating the shape of the concentration-time profile, with the same *f*AUC/MIC, against *P. aeruginosa* in 24-h static concentration time-kill experiments to address **Aim 1**. The findings in this chapter supported **Hypothesis 1** that the shape of the antibiotic concentration profiles at the same *f*AUC/MIC plays a role in determining the success of bacterial killing and resistance suppression for fluoroquinolones**.** The same *f*AUC/MIC delivered over a short duration (*i.e*. 1, 4 or 10 h) was compared with a longer duration (*i.e*. 16 or 24 h). This was tested with three *f*AUC/MIC exposure levels, one below and two above the threshold described for bacterial killing and clinical success of ciprofloxacin, against two inocula, one that had a high probability of containing one resistant mutant and the other that very likely contained no resistant mutants. The contribution of efflux pump related fluoroquinolone resistance was also assessed through the utilisation of an efflux pump inhibitor. It was observed that short-course, high-concentration ciprofloxacin suppressed resistance emergence, however regrowth was not suppressed. 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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Shape does matter: short high-concentration exposure minimizes resistance emergence for fluoroquinolones in *Pseudomonas aeruginosa*

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Objectives: For fluoroquinolones, the area under the free plasma concentration–time curve divided by the MIC (*f*AUC/MIC) best predicts bacterial killing in mice and outcomes in patients. However, it is unknown whether the shape of the antibiotic concentration profile affects resistance emergence. Our objective was to compare killing and resistance between ciprofloxacin concentration profiles with different shapes at the same *f*AUC/MIC and identify the durations of ciprofloxacin exposure that minimize resistance emergence.

Methods: Static time–kill studies over 24 h using *Pseudomonas aeruginosa* ATCC 27853 assessed *f*AUC/MIC of 44 and 132 of ciprofloxacin (MIC_{CIP} = 0.25 mg/L) and *f*AUC/MIC of 22, 44 and 132 of ciprofloxacin plus an efflux pump inhibitor ($MIC_{CIP+EPI}$ = 0.031 mg/L) at initial inocula of 10^4, 10^5 and 10^6 cfu/mL. Ciprofloxacin was added at 0 h and rapidly removed at 1, 4, 10, 16 or 24 h. Mutant frequencies and MICs were determined at 24 h.

Results: High ciprofloxacin concentrations over 1–10 h yielded more rapid and extensive initial killing compared with 16 and 24 h exposures at the same *f*AUC/MIC. No resistance emerged for 1–10 h exposures, although regrowth of susceptible bacteria was extensive. Ciprofloxacin exposure over 24 h yielded less regrowth, but ciprofloxacin-resistant bacteria at 5× MIC amplified by over 5 $\log_{10}$ and almost completely replaced the susceptible bacteria by 24 h; MICs increased 4- to 8-fold. Resistance also emerged on 3× MIC, but not 5× MIC, plates when efflux was inhibited.

Conclusions: Pre-existing resistant subpopulations amplified extensively with 24 and 16 h exposures, but not with shorter durations. The shape of the ciprofloxacin concentration profile was critical to minimize resistance emergence.

Keywords: ciprofloxacin, antibiotic resistance, *P. aeruginosa*, pharmacokinetic/pharmacodynamic relationships

Introduction

Pseudomonas aeruginosa and other Gram-negative pathogens are causing a global health crisis that is exacerbated by a severe shortage of effective antibiotics.[1–3] *P. aeruginosa* causes life-threatening infections in hospitalized patients and has an exceptional potential to become resistant during antibiotic therapy.[4–6] The most important resistance mechanisms for fluoroquinolones in *P. aeruginosa* include Mex efflux pumps[7,8] and target site mutations of *gyrA* and *parC*.[9]

Over the last few decades, extensive pharmacokinetic/pharmacodynamic (PK/PD) studies on fluoroquinolones have shown that the clinical success in patients and the bacterial killing at 24 h in mice are best predicted by the area under the free plasma concentration–time curve divided by the MIC (*f*AUC/MIC).[10–14] Forrest *et al.*[15] showed that a ciprofloxacin AUC/MIC >125 (equivalent to an *f*AUC/MIC of 87.5) is correlated with clinical success in acutely ill patients with bacterial infections, while at lower ratios the probability of clinical success was significantly decreased. However, this *f*AUC/MIC target was often not reached for strains with MICs of ≥1 mg/L.[16]

Dose fractionation studies are commonly performed to identify the PK/PD index that best predicts bacterial killing in mice and dynamic *in vitro* models. These studies divide a range of daily

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doses into one or multiple dose(s) using different dosing intervals. In dose fractionation studies, dosing continues throughout the 24 h period (except for once-daily dosing) and therefore antibiotic concentrations are present throughout the entire treatment period. Thus, dose fractionation studies do not identify the durations of antibiotic exposure that lead to resistance emergence at a given *f*AUC/MIC.

The *f*AUC and the free peak concentration (fC_{max}) are often correlated in patients and the fC_{max}/MIC usually also predicts bacterial killing in mice and clinical success of fluoroquinolones reasonably well.[12,14,17] Earlier studies suggested the need for future studies that identify whether dosage regimens with high fluoroquinolone peak concentrations better prevent clinical emergence of resistance than dosage regimens with lower peaks.[18,19] However, it is still not known whether high fluoroquinolone concentrations applied over a short period yield more or less resistance than lower concentrations over a longer period at the same overall exposure (i.e. at the same *f*AUC/MIC). A few studies determined the *f*AUC/MIC value that is associated with prevention of resistance for fluoroquinolones in *P. aeruginosa*.[20–23] However, it is unknown for fluoroquinolones which exposure durations lead to resistance at a given *f*AUC/MIC.

The primary objective of this study was to compare bacterial killing and resistance between ciprofloxacin concentration profiles at the same *f*AUC/MIC, but with different shapes, and to identify the durations of ciprofloxacin exposure that minimize resistance. Our second objective was to assess whether resistance emergence for the tested ciprofloxacin exposure profiles was dependent on efflux mechanisms. To achieve these objectives, we developed an appropriate study design based on static *in vitro* time–kill studies to evaluate resistance for different exposure profiles. Studies were performed at an initial inoculum of 10^6 cfu/mL, which most likely contained pre-existing resistant mutants, as well as at a low initial inoculum (10^4 cfu/mL), which most likely lacked such pre-existing mutants.

Materials and methods

Bacterial strains and media

The *P. aeruginosa* ATCC 27853 strain was used in all experiments. All susceptibility and time–kill studies were performed in cation-adjusted Mueller–Hinton broth (CAMHB; containing 20–25 mg/L Ca^{2+} and 10–12.5 mg/L Mg^{2+}; BD, Sparks, MD, USA). Viable counting was performed on cation-adjusted Mueller–Hinton agar (CAMHA; containing 25 mg/L Ca^{2+} and 12.5 mg/L Mg^{2+}; Medium Preparation Unit, University of Melbourne, Parkville, Australia). Drug-containing agar plates were prepared using CAMHA (BD, Sparks, MD, USA) supplemented with the appropriate amount of ciprofloxacin.

Ciprofloxacin was purchased from Sigma-Aldrich (Shanghai, China) and the efflux pump inhibitor PABN from Bachem (Bubendorf, Switzerland). Antibiotic stock solutions were prepared in Milli-Q water and subsequently filter-sterilized using a 0.22 μm PVDF syringe filter (Merck Millipore, Cork, Ireland).

Time–kill experiments

Static time–kill experiments were performed to assess bacterial killing and emergence of resistance for different ciprofloxacin exposure profiles with and without PABN. Bacteria were grown on CAMHA and incubated at 35°C for ∼20 h. Bacteria were then transferred into sterile CAMHB and incubated for 60 min in a shaking waterbath at 35°C. The optical density of this bacterial suspension was measured via a spectrophotometer to appropriately dilute this suspension to achieve the targeted initial inocula in 20 mL of fresh, pre-warmed, sterile CAMHB. The diluted bacterial suspensions were incubated in a shaking waterbath at 35°C for ∼15 min before the addition of ciprofloxacin, PABN or both.

We studied ciprofloxacin *f*AUC/MIC of 44 and 132 at initial inocula of 10^6, 10^5 and 10^4 cfu/mL. A ciprofloxacin *f*AUC/MIC of 87.5 represents the PK/PD breakpoint for clinical and microbiological cure.[15] For garenoxacin, an *f*AUC/MIC of 48 yielded extensive resistance emergence and an *f*AUC/MIC of 190 prevented resistance of *P. aeruginosa*.[21] We studied an *f*AUC/MIC of 44 to obtain extensive resistance and of 132 to achieve extensive killing with limited resistance. An *f*AUC/MIC of 22 was additionally studied to account for the potentially diminished capacity of *P. aeruginosa* to become resistant in the presence of efflux pump inhibition. A range of *f*AUC/MIC from ∼25 to 100 is relevant for clinical ciprofloxacin dosage regimens at the highest treatable MICs for *P. aeruginosa* of 0.25 and 0.5 mg/L. These exposures were achieved by the appropriate ciprofloxacin concentration applied over 1, 4, 10, 16 and 24 h. Ciprofloxacin was dosed at 0 h and then rapidly removed at the respective timepoint (removal procedure described below).

For studies of ciprofloxacin in combination with 60 mg/L PABN, we decreased the ciprofloxacin concentrations according to the MIC ratio with and without the efflux pump inhibitor. Thus, lower ciprofloxacin concentrations were used to achieve the targeted *f*AUC/MIC. Ciprofloxacin *f*AUC/MIC of 22, 44 and 132 in combination with 60 mg/L PABN were studied at initial inocula of 10^6 and 10^4 cfu/mL. The ciprofloxacin exposure durations were 1, 4, 10, 16 and 24 h. All studies included a growth control and a 60 mg/L PABN control without ciprofloxacin. The concentrations and exposure durations that were studied are listed in Table 1, both for studies without and with PABN.

Ciprofloxacin or ciprofloxacin and PABN were rapidly removed by multiple sequential centrifugation and resuspension steps. For vials with ciprofloxacin concentrations of ≥8× MIC, three sequential centrifugation and resuspension processes were used; for lower concentrations, two sequential steps were applied. The conical vials containing the bacterial cultures in broth were centrifuged at 3220 **g** for 10 min at 35°C, the supernatant removed and the bacteria resuspended in fresh, pre-warmed, drug-free CAMHB. The overall drug dilution factor was ∼400-fold for two sequential centrifugation and resuspension processes and 8000-fold for three processes. This method assured that the ciprofloxacin concentrations were negligible (<0.004× MIC) after drug removal.

Viable counting

For all experimental arms, counts of viable bacteria were determined within 5 min prior to dosing and at 1, 2, 3.5 or 3.9, 6, 12, 16.5 and 24 h after dosing. Bacterial numbers were also determined 5 min prior to drug removal and 10 min after the final bacterial resuspension in fresh broth to assure minimal loss of bacteria during drug removal. All viable count samples were washed twice in sterile saline to effectively minimize antibiotic carryover. Colony counts on CAMHA were determined by manual plating of 100 μL of the undiluted or diluted bacterial suspensions in saline. This plating method yielded a limit of counting of 1.0 $\log_{10}$ cfu/mL (equivalent to one colony per plate). Agar plates were incubated at 35°C for 48 h.

Emergence of resistance

Mutant frequencies (MFs) were determined at 0 h (i.e. before treatment) and at 24 h to determine the abundance of resistant bacteria in the population before and after treatment. MICs were determined at 24 h by spectrophotometrically adjusting the bacterial suspensions (i.e. dilution in fresh, pre-warmed, sterile CAMHB) to an inoculum of 10^6 cfu/mL if the

Table 1. Static concentrations and exposure durations of ciprofloxacin for each studied *f*AUC/MIC

	Ciprofloxacin concentration (mg/L)		Ciprofloxacin concentration (mg/L) plus 60 mg/L PABN[d]		
Drug exposure duration (h)	*f*AUC/MIC: 44[a]	*f*AUC/MIC: 132[b]	*f*AUC/MIC: 22[c]	*f*AUC/MIC: 44[b]	*f*AUC/MIC: 132[c]
Control	0	0	0	0	0
1	11	33	0.688	1.38	4.13
4	2.75	8.25	0.172	0.344	1.03
10	1.1	3.3	0.069	0.138	0.41
16	0.69	2.06	0.043	0.086	0.26
24	0.46	1.38	0.029	0.057	0.172

The ciprofloxacin MIC was 0.25 mg/L without PABN and 0.031 mg/L with 60 mg/L PABN.
[a]Studied initial inocula: 10^4, 10^5 and 10^6 cfu/mL.
[b]Studied initial inocula: 10^4 and 10^6 cfu/mL.
[c]Studied initial inoculum: 10^6 cfu/mL.
[d]Control arms were studied with and without PABN.

bacterial suspension was $>10^6$ cfu/mL. For experimental arms with $<10^6$ cfu/mL at 24 h, undiluted bacterial suspensions were used for MIC testing at 24 h. Ciprofloxacin was removed from all arms before testing emergence of resistance.

Agar plates containing 3× or 5× the ciprofloxacin MIC were used for studies on ciprofloxacin monotherapy. For studies on ciprofloxacin with 60 mg/L PABN, the ciprofloxacin concentration in agar was calculated based on the lower MIC in the presence of PABN and drug plates contained 3× or 5× this lower ciprofloxacin MIC plus 60 mg/L PABN. Antibiotic-containing agar plates were incubated for 3 days and MF calculated as the difference between the $\log_{10}$ cfu/mL on antibiotic-containing agar plates and the $\log_{10}$ cfu/mL on drug-free plates at the same observation time.

Some of the viable counts at 24 h were too low to enable quantifying colonies on antibiotic-containing agar plates. These arms still provided information on the upper limit of the $\log_{10}$ MF (such as $\log_{10}$ MF of -6 or less). To include these data in the summary statistics, we used the following reporting rules. If the calculated MF was not quantifiable, but the upper limit was within 1.1 $\log_{10}$ of the MF for the growth control, we assumed the MF was unchanged and used the value of the growth control. If the MF was not quantifiable and the upper limit was >1.1 $\log_{10}$ higher than the MF for the growth control, the MF of this arm was reported as missing.

Results

The MIC of ciprofloxacin for *P. aeruginosa* ATCC 27853 was 0.25 mg/L. The $\log_{10}$ mutation frequency before treatment was -6.2 on 3× MIC ciprofloxacin plates and -7.1 on 5× MIC ciprofloxacin plates. As expected, the extent of killing of *P. aeruginosa* increased with the ciprofloxacin exposure (*f*AUC/MIC). Ciprofloxacin without PABN at *f*AUC/MIC of 44 and 132 yielded ∼2 to >5 $\log_{10}$ killing (Figure 1). At the *f*AUC/MIC of 44, initial killing was noticeably slower for the 16 and 24 h durations of exposure than for the shorter durations. Initial killing was rapid for all durations of exposure at the *f*AUC/MIC of 132 with minimum viable counts occurring within the first 4 h.

Considerable regrowth at 24 h was observed for the vast majority of ciprofloxacin profiles, in particular at the 10^5 and 10^6 cfu/mL inocula. Complete killing with no regrowth was observed for the 4 h duration of exposure at the *f*AUC/MIC of 132 for the 10^4 cfu/mL inoculum. Based on our MF results, the 10^4 cfu/mL inoculum had a probability of ∼1.2% to harbour at least one pre-existing mutant cell resistant at 5× MIC; the probability was ∼10% at 3× MIC. In contrast, the 10^6 cfu/mL inoculum had a probability >99.99% to contain at least one pre-existing resistant mutant at 3× MIC and a probability of ∼80% to contain a resistant mutant at 5× MIC. Overall regrowth was slower and viable counts at 24 h tended to be lower for the 24 h duration of exposure compared with the shorter durations (Figure 1).

The extensive regrowth at 24 h for almost all viable count profiles posed the important question of whether these bacteria were resistant to ciprofloxacin. Interestingly, for the 24 h ciprofloxacin exposure, the MF on 3× MIC plates was ∼6.1 $\log_{10}$ (at the *f*AUC/MIC of 44) and 5.6 $\log_{10}$ (at the *f*AUC/MIC of 132) higher than the MF of the growth control at 24 h (Figure 2). Likewise, a 5.9 $\log_{10}$ (at the *f*AUC/MIC of 44) and 7.3 $\log_{10}$ (at the *f*AUC/MIC of 132) higher MF on 5× MIC plates was observed. This trend was clearly present for both the 10^6 and 10^5 cfu/mL inocula. Ciprofloxacin over a 16 h exposure duration at the *f*AUC/MIC of 44 yielded up to 6.5 $\log_{10}$ increased MF on 3× and 5× MIC plates, whereas the MF was increased by up to 2.6 $\log_{10}$ for the *f*AUC/MIC of 132 at the 10^6 cfu/mL inoculum.

Emergence of resistance, however, did not occur or was substantially less for the shorter durations of ciprofloxacin exposure (1, 4 and 10 h). The MF for these durations of exposure was comparable to the MF of the growth control at 24 h (only up to 1.9 $\log_{10}$ higher) for all studied inocula on 3× and 5× MIC plates (Figure 2).

The higher MF correlated well with the increased MICs at 24 h (Table 2). The MICs remained relatively unchanged for 1–10 h durations of exposure, whereas the MICs increased up to 8-fold at the 10^6 and 10^5 cfu/mL inocula for the 16 and 24 h durations of exposure (Table 2). Emergence of resistance at the 10^4 cfu/mL inoculum was much less compared with the 10^6 and 10^5 cfu/mL inocula (Figure 2 and Table 2).

To explore whether efflux was the primary cause for emergence of resistance, we carried out time–kill experiments in the presence of 60 mg/L PABN (Figure 3). The ciprofloxacin MIC was 0.031 mg/L in the presence of 60 mg/L PABN. Thus, 8-fold lower ciprofloxacin concentrations were used to account for the lower MIC in the presence of PABN. The $\log_{10}$ mutation frequency before treatment was -5.4 on 3× MIC ciprofloxacin with PABN plates and was -8.2 on 5× MIC ciprofloxacin with

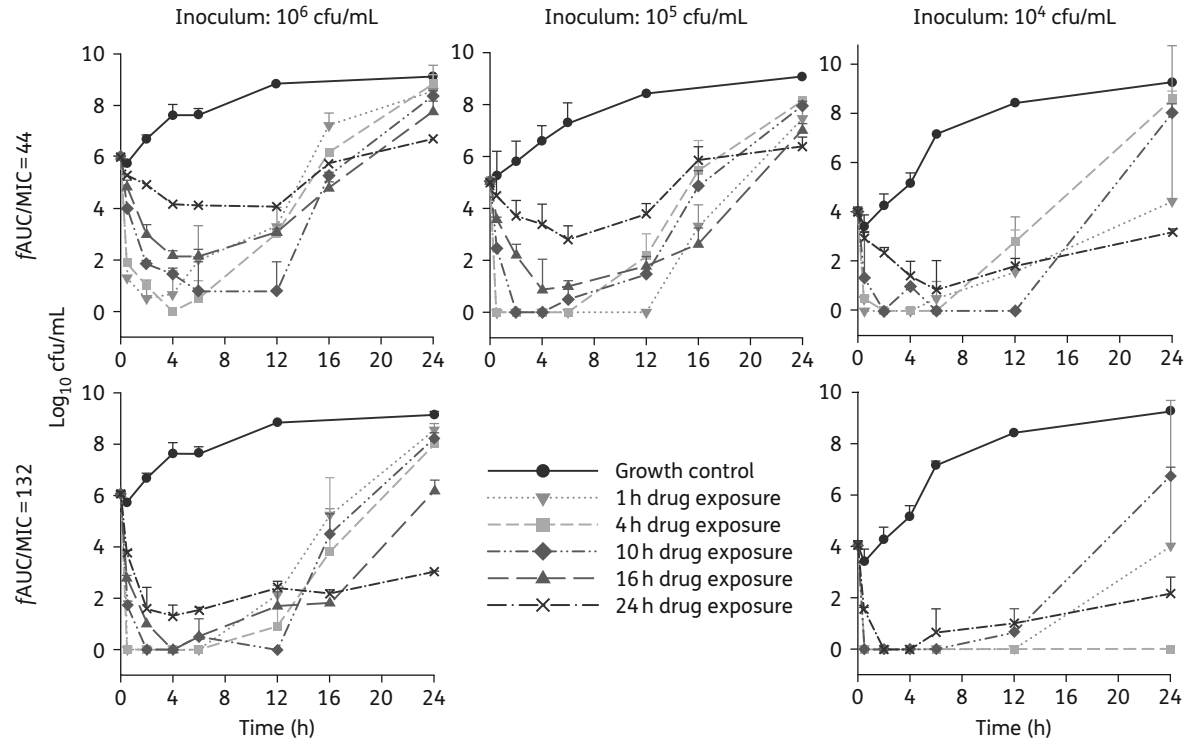

Figure 1. Observed viable counts (mean ± SD) for *P. aeruginosa* ATCC 27853 exposed to ciprofloxacin at *f*AUC/MIC of 44 (top) or 132 (bottom). The same *f*AUC/MIC (44 or 132) of ciprofloxacin were delivered by varying the duration of exposure over 1, 4, 10, 16 or 24 h at inocula of 10^6 (left), 10^5 (middle) and 10^4 cfu/mL (right). The 16 h exposure was not studied at the 10^4 cfu/mL inoculum. None of these treatment arms contained PABN. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

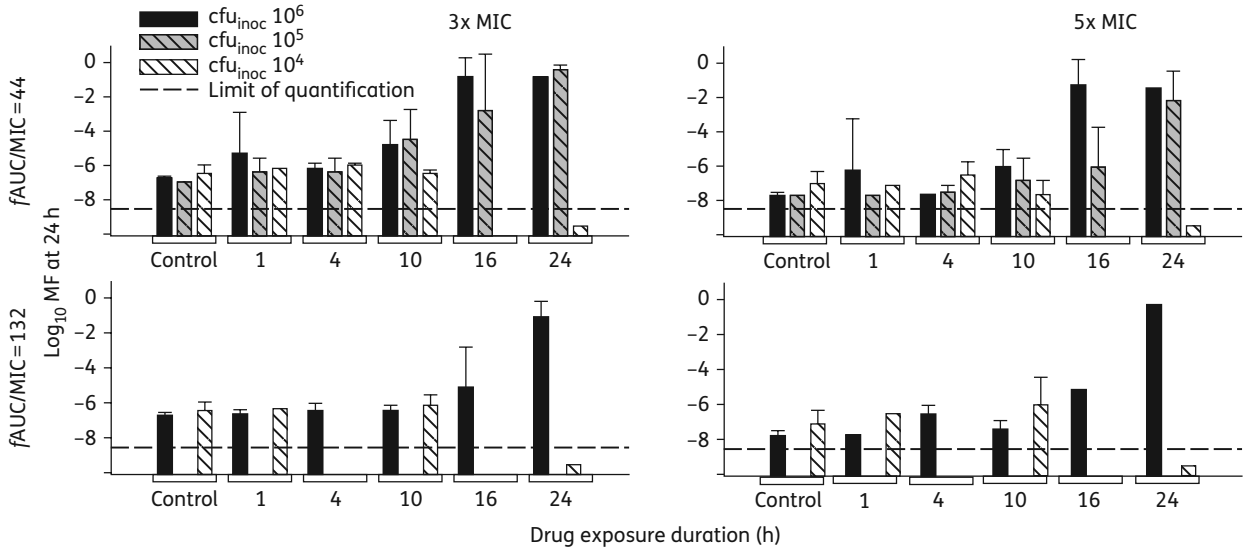

Figure 2. Log_{10} MF (mean ± SD) at 24 h on 3× (left) and 5× MIC (right) agar plates for ciprofloxacin in the absence of PABN at *f*AUC/MIC of 44 (top) or 132 (bottom) delivered over differing durations of exposure. Inocula (cfu_{inoc}) of 10^6, 10^5 and 10^4 cfu/mL were studied for the *f*AUC/MIC of 44 and inocula of 10^6 and 10^4 cfu/mL for the *f*AUC/MIC of 132. The *f*AUC/MIC of 132 yielded eradication for the 4 h exposure duration at the 10^4 cfu/mL inoculum and thus no MF could be determined. The 16 h duration of exposure was not studied at the 10^4 cfu/mL inoculum. The MF for the 24 h exposure at the 10^4 cfu/mL inoculum was arbitrarily drawn at -9.5 log_{10} (i.e. below the limit of quantification); these two experimental arms had $<10^{3.3}$ cfu/mL for the total population at 24 h and no colonies grew on antibiotic-containing agar plates.

Table 2. MICs (mg/L) at 24 h [geometric mean (range)] for ciprofloxacin *f*AUC/MIC of 44 or 132 delivered over various durations of exposure and initial inocula (cfu_{inoc})

	*f*AUC/MIC: 44			*f*AUC/MIC: 132	
Drug exposure duration (h)	cfu_{inoc} 1×10^6	cfu_{inoc} 1×10^5	cfu_{inoc} 1×10^4	cfu_{inoc} 1×10^6	cfu_{inoc} 1×10^4
Control	0.25 (0.25–0.25)	0.50	0.25 (0.25–0.25)	0.25 (0.25–0.25)	0.25 (0.25–0.25)
1	0.50 (0.25–**1.00**)	0.35 (0.25–0.50)	0.25	0.25 (0.25–0.25)	0.50
4	0.25 (0.25–0.25)	0.35 (0.25–0.50)	0.25 (0.25–0.25)	0.25 (0.25–0.25)	0.25
10	0.50 (0.25–**1.00**)	0.71 (0.50–**1.00**)	0.25 (0.25–0.25)	0.25 (0.25–0.25)	0.35 (0.25–0.50)
16	**2.00** (**2.00–2.00**)	0.71 (0.50–**1.00**)	not studied	0.50 (0.25–**1.00**)	not studied
24	**1.41** (**1.00–2.00**)	**1.00** (**1.00–1.00**)	0.18 (0.125–0.25)	**1.41** (**1.00–2.00**)	0.25

MICs are in bold if they were ≥4-fold above baseline. No range is provided if only one replicate was available.

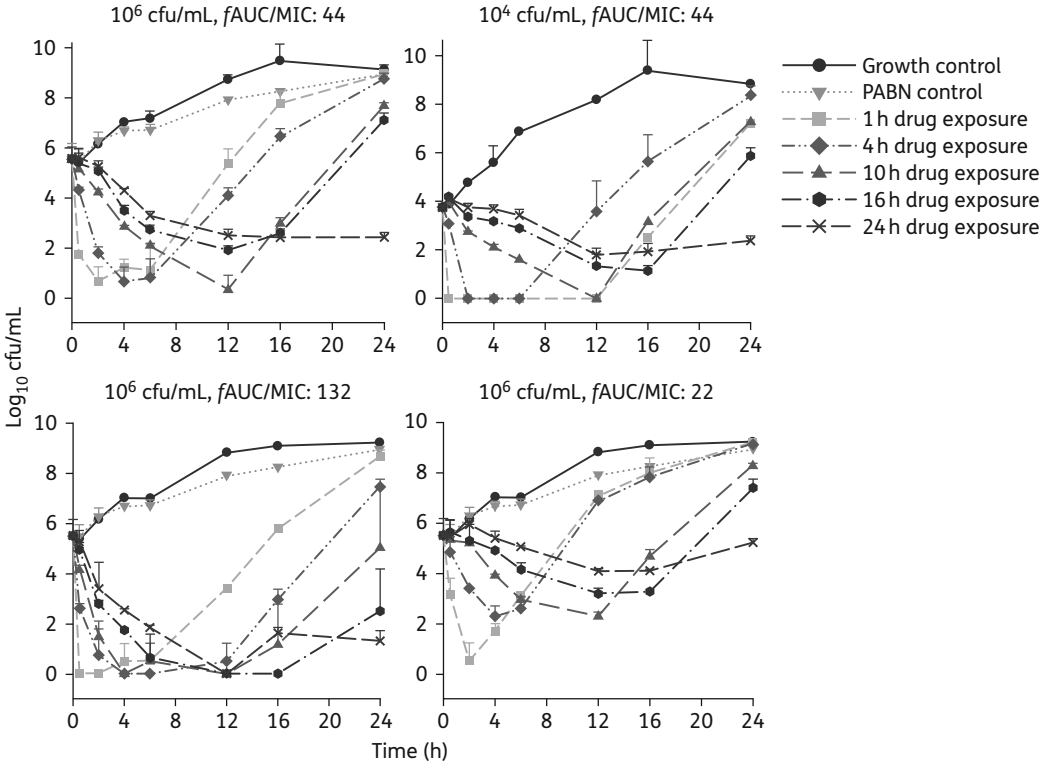

Figure 3. Observed viable counts (mean ± SD) for *P. aeruginosa* ATCC 27853 exposed to ciprofloxacin, *f*AUC/MIC of 22, 44 or 132, in combination with 60 mg/L PABN. The same *f*AUC/MIC (22, 44 or 132) of ciprofloxacin were delivered by varying the duration of exposure over 1, 4, 10, 16 or 24 h at initial inocula of 10^6 and 10^4 cfu/mL. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

PABN plates. Viable count profiles for 60 mg/L PABN largely paralleled the growth control. In the presence of PABN, all experimental arms achieved >5 $\log_{10}$ killing at the ciprofloxacin *f*AUC/MIC of 132. At the *f*AUC/MIC of 22 and 44, the maximum extent of killing was larger and minimum viable counts occurred earlier for the shorter compared with the longer durations of exposure (Figure 3). Even in the presence of 60 mg/L PABN, ciprofloxacin could not eradicate *P. aeruginosa*. Regrowth was limited or absent for the 24 h duration of exposure at all studied *f*AUC/MIC, but regrowth occurred for the shorter durations of exposure.

For ciprofloxacin combined with 60 mg/L PABN, 10 and 16 h durations of exposure led, in general, to considerably increased MF at 24 h on 3× MIC plates compared with the MF of the growth control at 24 h (Figure 4). Resistance emergence was absent or much less for the 1 and 4 h durations of exposure. Viable counts for the 24 h duration of exposure were low at 24 h and the MF was usually below the quantification limit for these arms. Resistance on 3× MIC plates was similar for ciprofloxacin with and without the efflux pump inhibitor (Figures 2 and 4); except for the 10 h duration of exposure for the *f*AUC/MIC of 132 that showed an ~6.4 $\log_{10}$ increase in MF in the presence of PABN (Figure 4).

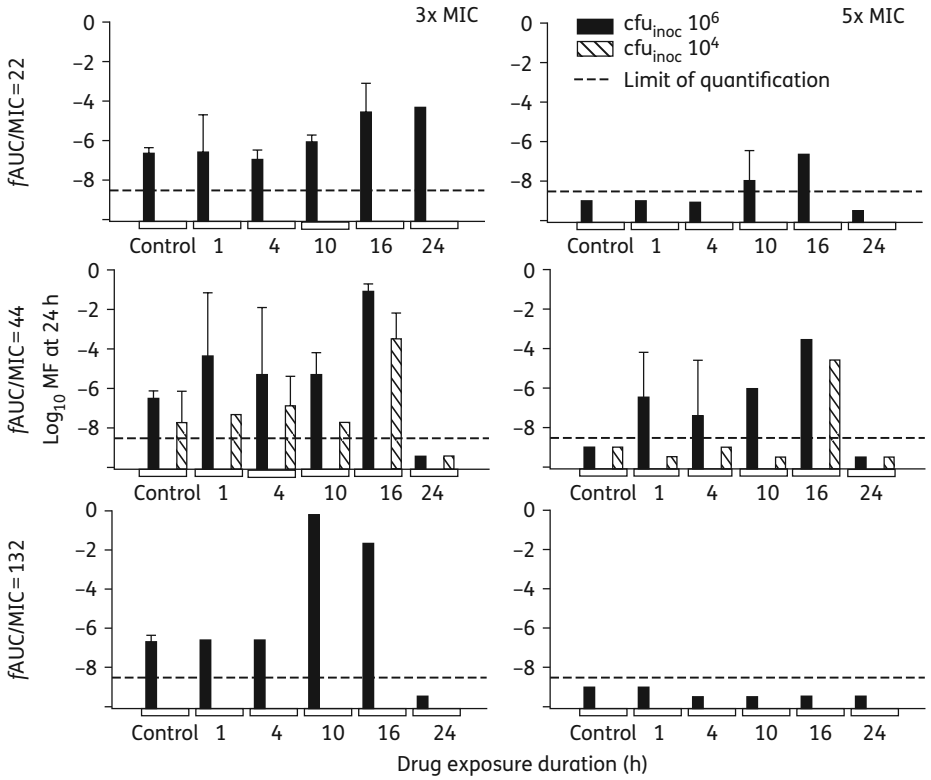

Figure 4. Log_{10} MF (mean ± SD) at 24 h on 3× (left) and 5× MIC (right) plates for ciprofloxacin in the presence of 60 mg/L PABN at *f*AUC/MIC of 22 (top), 44 (middle) or 132 (bottom) delivered over differing durations of exposure. Inocula (cfu_{inoc}) of 10^6 and 10^4 cfu/mL were studied for the *f*AUC/MIC of 44 and an inoculum of 10^6cfu/mL for *f*AUC/MIC of 22 and 132. The growth controls in the presence of 60 mg/L PABN did not yield colonies on agar plates with 5× MIC; given their total viable counts of $\sim 10^9$ cfu/mL, the log_{10} MF of the growth controls for 5× MIC is drawn as −9 (below the limit of quantification). For all experimental arms that had no colonies on agar plates containing 5× MIC ciprofloxacin and that had a viable count of $\geq 10^{7.9}$ cfu/mL on antibiotic-free agar plates, the log_{10} MF was drawn as −9. For experimental arms that showed no colonies on antibiotic-containing agar plates that had total populations of $<10^{7.9}$ cfu/mL on antibiotic-free agar plates, the log_{10} MF was arbitrarily drawn at −9.5 (below the limit of quantification).

Table 3. MICs (mg/L) at 24 h [geometric mean (range)] for ciprofloxacin *f*AUC/MIC of 22, 44 or 132 delivered over various durations of exposure and initial inocula (cfu_{inoc}) in the presence of 60 mg/L PABN

	*f*AUC/MIC: 22	*f*AUC/MIC: 44		*f*AUC/MIC: 132
Drug exposure duration (h)	cfu_{inoc} 1×10^6	cfu_{inoc} 1×10^6	cfu_{inoc} 1×10^4	cfu_{inoc} 1×10^6
Control	0.025 (0.016–0.031)	0.025 (0.016–0.031)	0.031 (0.031–0.031)	0.025 (0.016–0.031)
PABN control	0.022 (0.016–0.031)	0.022 (0.016–0.031)	not studied	0.022 (0.016–0.031)
1	0.031 (0.031–0.031)	0.063 (0.031–**0.25**)	0.044 (0.031–0.063)	0.022 (0.016–0.031)
4	0.031 (0.031–0.031)	0.050 (0.031–**0.125**)	0.044 (0.031–0.063)	0.031 (0.031–0.031)
10	0.022 (0.016–0.031)	0.031 (0.031–0.031)	0.031	0.062 (0.016–**0.25**)
16	0.022 (0.016–0.031)	**0.25** (**0.25–0.25**)	0.063	**0.125**
24	0.063 (0.063–0.063)			

MICs are in bold if they were ≥4-fold above baseline. No range is provided if only one replicate was available.

However, resistance on 5× MIC plates was less in the presence of PABN compared with the absence of PABN. Emergence of resistance for the 16 and 24 h durations of exposure also occurred for an *f*AUC/MIC of 22, but was less pronounced at this low ciprofloxacin *f*AUC/MIC.

The MF corresponded with the MICs of the combination of ciprofloxacin with 60 mg/L PABN (Table 3). MICs were relatively unchanged except for the 16 h duration of exposure, which led to a 5- to 10-fold increased MIC for *f*AUC/MIC of 44 or 132 at the 10^6 cfu/mL inoculum (Table 3). The MICs at 24 h could not

be determined for the 24 h duration of exposure for *f*AUC/MIC of 44 and 132, probably due to the low viable counts.

Discussion

Many PK/PD studies on fluoroquinolones have found the *f*AUC/MIC to be the PK/PD index that best predicts bacterial killing in mice and therapeutic success in patients.[11,13–15] From such studies conducted during drug development, clinical dosing of fluoroquinolones is now guided by the *f*AUC/MIC. However, only a few studies assessed prevention of resistance to fluoroquinolones against Gram-negative pathogens.[20–23] While the design of these studies implicitly assumed that the *f*AUC/MIC best predicts prevention of resistance for fluoroquinolones, it is unknown which PK/PD index is relevant for this endpoint. In fact, for rifampicin and linezolid, different PK/PD indices predict bacterial killing (*f*AUC/MIC) and prevention of resistance (fC_{max}/MIC).[24,25]

In the present study, we demonstrated that emergence of resistance was more prominent (>5 $\log_{10}$) at longer durations of exposure (16 and 24 h) than shorter durations at the same *f*AUC/MIC (Figure 2 and Table 2). A short duration of exposure yielded more killing at 2–4 h than long durations of exposure at the same *f*AUC/MIC (Figure 1). At the 10^4 cfu/mL inoculum, no or little emergence of resistance was present and MICs were not elevated, most likely due to lack of pre-existing resistant mutants (Figure 2 and Table 2). It seems possible that the high ciprofloxacin concentrations during the 1–10 h durations of exposure killed both susceptible and resistant bacteria to a comparable extent and therefore did not give rise to emergence of resistance. In contrast, the 24 h duration of exposure probably provided a growth advantage for resistant mutants. Alternatively, adaptive resistance, if present, might require durations of antibiotic exposure of >10 h to be (fully) up-regulated or adaptive resistance may revert back to baseline between 10 and 24 h. The latter two alternatives seem less likely, as efflux of levofloxacin in *P. aeruginosa* was extensively up-regulated within 1 h and did not revert back to baseline between 6 and 24 h, i.e. when levofloxacin concentrations were negligible due to a short half-life and a 24 h dosing interval in mice.[22]

We also considered the possibility that the emergence of resistance for longer durations of ciprofloxacin exposure may have been due to induction of the SOS response.[26,27] However, we observed considerably less emergence of resistance in the 10^4 (unlike the 10^6) cfu/mL inoculum at the same *f*AUC/MIC, which suggests resistance was at least in part caused by pre-existing resistant mutants. Furthermore, antibiotic treatment has been proposed to cause oxidative stress, which, when this stress is only small, allows beneficial mutations and therefore emergence of resistance to occur instead of bacterial killing.[28] However, as discussed above, most of the resistance emergence observed in the present study may probably have been due to pre-existing resistant mutants. Ultimately, molecular studies combined with a full mechanism-based modelling analysis would be highly valuable to elucidate these mechanistic details.

We explored the role of efflux in the observed rapid and extensive emergence of resistance. In the presence of the broad-spectrum efflux pump inhibitor PABN, which inhibits the most relevant efflux pumps for fluoroquinolones (MexAB, MexCD and MexEF),[7] considerable emergence of resistance occurred for *f*AUC/MIC of 44 and 132 for the 16 h duration of exposure (Figure 4 and Table 3). Emergence of resistance was less pronounced at the *f*AUC/MIC of 22, probably because this drug exposure did not provide a sufficient growth advantage for less susceptible bacteria, in agreement with the inverted-U principle[20,21] and the general concept of the mutant selection window.[29] As emergence of resistance on 5× MIC plates in the presence of PABN was considerably less (Figure 4) than in the absence of PABN (Figure 2), efflux played a role in the development of high-level resistance.

The extensive contribution of efflux pumps to fluoroquinolone resistance was studied in detail in a 48 h mouse infection model at a high bacterial inoculum; an *f*AUC/MIC of 110 for levofloxacin (equivalent to a total drug AUC/MIC of 157) prevented amplification of resistant mutants whereas an *f*AUC/MIC of 37 led to amplification of resistant mutants.[22] Two hollow fibre *in vitro* infection model studies determined the *f*AUC/MIC of garenoxacin that prevented resistance;[20,21] for *P. aeruginosa*, an *f*AUC/MIC of 190 prevented amplification of pre-existing resistant mutants at 48 h.[21] Another hollow fibre study found extensive emergence of resistance to ciprofloxacin by 48–72 h using a ciprofloxacin *f*AUC/MIC of 180 against three *P. aeruginosa* strains.[23] The studies mentioned above[20–23] assessed emergence of resistance for different dose levels and thus for different *f*AUC/MIC. These studies neither varied the fluoroquinolone half-life nor the dosing interval and therefore kept the shape of the antibiotic concentration–time profile constant.

An earlier dose fractionation study in the hollow fibre model with ciprofloxacin against two *P. aeruginosa* isolates found extensive resistance emergence for 400 mg of ciprofloxacin dosed every 8 h and 600 mg every 12 h (equivalent to an *f*AUC/MIC of ∼60).[19] 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 this regimen was subject to regrowth of mostly susceptible *P. aeruginosa* at 24 h.[19] Another *in vitro* study gave the same daily enoxacin dose (equivalent to an *f*AUC/MIC of ∼50) every 12 or 24 h and found more killing for dosing every 24 h. However, extensive *P. aeruginosa* resistance occurred at 12–24 h for both regimens.[30] These dose fractionation studies[19,30] had high or low fluoroquinolone concentrations present throughout the entire treatment period. Therefore, the durations of fluoroquinolone exposure that lead to resistance have not been assessed previously.[19–23,30]

The present study systematically explored whether the shape of the ciprofloxacin concentration–time profile affects bacterial killing and resistance emergence. We developed an appropriate *in vitro* study design that delivered the same *f*AUC/MIC by varying the duration of exposure and concentration of ciprofloxacin (and the efflux pump inhibitor where applicable) accordingly. Most available studies on emergence of fluoroquinolone resistance in dynamic *in vitro* or animal models were performed at high inocula that contained pre-existing resistant mutants.[20–22] We extended these studies by assessing a low inoculum (10^4 cfu/mL) in duplicate that most likely (probability ≥90%) lacked pre-existing resistant mutants.

Our study was designed to achieve the objectives of this work; a potential limitation is the use of static antibiotic concentrations. Therefore, future studies in dynamic *in vitro* and animal infection models are warranted to further confirm that short durations of exposure provide killing with no or limited resistance. Our study lacked the effect of the immune system and used standard,

nutrient-rich broth medium. These factors probably resulted in more rapid (re)growth of bacteria compared with an *in vivo* infection, in agreement with the relatively slow regrowth of *P. aeruginosa* in mice.[22]

In summary, we found that delivering the same *f*AUC/MIC over short durations of exposure (i.e. 1, 4 or 10 h) achieved more rapid killing with no or very limited emergence of resistance, whereas longer durations of exposure over 16 and 24 h led to a dramatic (5 $\log_{10}$) increase in the concentration of resistant bacteria. Therefore, the shape of the concentration–time profile had a pronounced effect on prevention of resistance emergence. Pre-existing resistant mutants probably caused emergence of resistance. Efflux was important for the development of high-level resistance (at 5× MIC), but was not required for the development of low-level resistance on 3× MIC plates. Regrowth of *P. aeruginosa* was extensive for most regimens with durations of exposure of up to 16 h, indicating that the post-antibiotic effect of ciprofloxacin was short.[31] Therefore, clinical ciprofloxacin regimens with high-intensity, short-exposure durations may provide extensive and rapid bacterial killing with no or limited resistance. They would be expected to be best used as part of a combination regimen with a second antibiotic that prevents regrowth of ciprofloxacin-susceptible bacteria. Studies in dynamic *in vitro* and animal infection models are warranted to optimally translate our results to future studies in patients. The present study highlights the potential to greatly minimize emergence of resistance by innovative fluoroquinolone dosage regimens with an optimized shape of the plasma concentration–time profiles.

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Transparency declarations

None to declare.

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Chapter 3

Resistance suppression by high-intensity, short-duration aminoglycoside exposure against hypermutable and non-hypermutable *Pseudomonas aeruginosa*

Preamble

This Chapter examined the aminoglycoside tobramycin in monotherapy against hypermutable and non-hypermutable *P. aeruginosa*. The *f*AUC/MIC target is widely recognised as one of the best PK/PD predictors of bacterial killing for aminoglycosides. **Aim 1** was addressed through evaluation of the shape of the tobramycin concentration-time profile, with the same *f*AUC/MIC in 24-h static concentration time-kill experiments and development of novel MBM. The findings in this chapter further supported **Hypothesis 1**, that the differently shaped antibiotic concentration profiles of an aminoglycoside at the same *f*AUC/MIC can determine the success of bacterial killing and resistance suppression. This chapter also addressed **Aim 2** in demonstrating that a hypermutable laboratory strain showed elevated emergence of resistance across different tobramcyin profiles. This suggested that hypermutator strains are likely more difficult to treat than non-hypermutable strains, thus supporting **Hypothesis 2.** Therefore, it was proposed that a second antibiotic in combination with tobramycin was required to improve its antibacterial effects, especially against hypermutable *P. aeruginosa*. This was the first study to evaluate the *f*AUC/MIC target with respect to which shape of the *f*AUC is most effective at suppressing the emergence of aminoglycoside resistance. 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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Resistance suppression by high-intensity, short-duration aminoglycoside exposure against hypermutable and non-hypermutable *Pseudomonas aeruginosa*

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Objectives: Hypermutable bacteria are causing a drastic problem via their enhanced ability to become resistant. Our objectives were to compare bacterial killing and resistance emergence between differently shaped tobramycin concentration–time profiles at a given *f*AUC/MIC, and determine the tobramycin exposure durations that prevent resistance.

Methods: Static concentration time–kill studies over 24 h used *Pseudomonas aeruginosa* WT strains (ATCC 27853 and PAO1) and hypermutable PAOΔ*mutS*. *f*AUC/MIC values of 36, 72 and 168 were assessed at initial inocula of 10^6 and 10^4 cfu/mL (all strains) and $10^{1.2}$ cfu/mL (PAOΔ*mutS* only) in duplicate. Tobramycin was added at 0 h and removed at 1, 4, 10 or 24 h. Proportions of resistant bacteria and MICs were determined at 24 h. Mechanism-based modelling was conducted.

Results: For all strains, high tobramycin concentrations over 1 and 4 h resulted in more rapid and extensive initial killing compared with 10 and 24 h exposures at a given *f*AUC/MIC. No resistance emerged for 1 and 4 h durations of exposure, although extensive regrowth of susceptible bacteria occurred. The 24 h duration of exposure revealed less regrowth, but tobramycin-resistant populations had completely replaced susceptible bacteria by 24 h for the 10^6 cfu/mL inoculum. The hypermutable PAOΔ*mutS* showed the highest numbers of resistant bacteria. Total and resistant bacterial counts were described well by novel mechanism-based modelling.

Conclusions: Extensive resistance emerged for 10 and 24 h durations of exposure, but not for shorter durations. The tobramycin concentration–time profile shape is vital for resistance prevention and should aid the introduction of optimized combination regimens.

Introduction

A global healthcare crisis is arising from Gram-negative bacteria, such as *Pseudomonas aeruginosa*, where effective antibiotics are increasingly scarce leading to difficult-to-treat life-threatening infections.[1–4] The occurrence of hypermutator phenotypes in *P. aeruginosa* clinical isolates is exacerbating the problem because of their enhanced ability to become resistant.[5,6] The fast-acting aminoglycoside tobramycin causes significant bacterial killing although extensive resistance may occur following monotherapy.[7] Tobramycin is a protein synthesis inhibitor[8] that has also been found to disrupt the bacterial outer membrane.[9,10] The most important resistance mechanisms of *P. aeruginosa* against aminoglycosides are the up-regulation of the MexXY-OprM efflux pump,[11–13] reduced permeability of the outer membrane, and enzymes that inactivate aminoglycosides intracellularly by phosphorylation, acetylation or adenylation.[14]

Hypermutable strains of *P. aeruginosa* are of major concern, particularly in chronic respiratory infections such as those in patients with cystic fibrosis (CF).[15–17] Hypermutation is caused by defects in DNA or error repair systems[18] and is most commonly due to mutations in the *mutS* gene, encoding a component of the

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methyl-directed mismatch repair system.[5,19,20] It provides bacteria with an advantage through the ability to adapt quickly to stressful and fluctuating environments via rapidly gaining or enhancing resistance mechanisms.[15,21,22]

Two pharmacokinetic/pharmacodynamic indices are most commonly used as predictors for bacterial killing by aminoglycosides, being the *f*AUC/MIC and the fC_{max}/MIC.[23–25] The fC_{max}/MIC relies on the concentration at a single timepoint within a dosage interval. The *f*AUC/MIC only considers the total (i.e. time-averaged) exposure across a 24 h period and suggests the same extent of bacterial killing regardless of the shape of the concentration–time profile. Once- and thrice-daily intravenous aminoglycoside therapy at the same daily dose were found to be equally effective clinically against pulmonary exacerbations of CF, but the emergence of resistance was not studied.[26,27] However, in a small clinical study in 33 patients with CF, tobramycin C_{max}/MIC was found as the best predictor of clinical outcome as measured by lung function;[28] the investigators suggested that resistance was greater after once-daily dosing, but an alternative analysis may have led to a different conclusion. Recently we have shown that high ciprofloxacin concentrations for a short duration of exposure (at a specific *f*AUC/MIC) resulted in resistance prevention.[29]

The main objective of the current investigation was to evaluate the bacterial killing and emergence of resistance resulting from differently shaped tobramycin concentration–time profiles at a given *f*AUC/MIC. We aimed to determine whether short-duration, high concentrations were more efficient in bacterial killing and prevention of the emergence of resistance compared with low concentrations over longer durations. Furthermore, we sought to evaluate resistance prevention in the worst-case scenario of a hypermutable strain. To address these objectives we used *in vitro* time–kill studies to assess bacterial killing and resistance emergence for different concentration–time profiles, at given *f*AUC/MIC in hypermutable and non-hypermutable *P. aeruginosa*.

Materials and methods

Bacterial strains and media

The *P. aeruginosa* ATCC 27853 and PAO1 WT reference strains were used in this study. We also used the isogenic hypermutable *P. aeruginosa* PAOΔ*mutS* strain that was constructed from the PAO1 WT reference strain by Mena *et al*.[30] via deletion of the *mutS* gene. All susceptibility and time–kill studies were performed in CAMHB (containing 20–25 mg/L Ca^{2+} and 10–12.5 mg/L Mg^{2+}; BD, Sparks, MD, USA). Viable counting was performed on cation-adjusted Mueller–Hinton agar (containing 25 mg/L Ca^{2+} and 12.5 mg/L Mg^{2+}; Medium Preparation Unit, The University of Melbourne, Parkville, Victoria, Australia). Drug-containing agar plates were prepared using cation-adjusted Mueller–Hinton agar (BD) supplemented with the appropriate amount of tobramycin (AK Scientific, Union City, MD, USA). The antibiotic stock solution was prepared in Milli-Q water and subsequently filter-sterilized using a 0.22 μm PVDF syringe filter (Merck Millipore, Cork, Ireland).

Time–kill experiments

To assess bacterial killing and emergence of resistance, time–kill experiments were performed in duplicate as previously described[29] for the different tobramycin exposure profiles. We studied three overall (i.e. time-averaged) tobramycin exposures, corresponding to *f*AUC/MIC of 36, 72 and 168; two exposures were above and one below the recommended *f*AUC/MIC exposure of 42 for the bactericidal effect.[24] For MICs ≤1 mg/L (83% of *P. aeruginosa* isolates reported by EUCAST),[31] the two lower exposures are achievable at common clinical doses, whereas *f*AUC/MIC of 168 requires a high clinical dose in ICU patients.[32] The tobramycin agar dilution MIC using the CLSI method[33] was 0.5 mg/L for PAO1 and ATCC 27853; while for PAOΔ*mutS* it was 1 mg/L due to the resistant bacterial subpopulations.[34]

Table 1. Tobramycin concentrations (mg/L) and durations of exposure for each studied *f*AUC/MIC against three strains, ATCC 27853 (MIC 0.5 mg/L), PAO1 (MIC 0.5 mg/L) and PAOΔ*mutS* (MIC 1 mg/L)

Drug exposure duration (h)	*f*AUC/MIC: 36	*f*AUC/MIC: 72	*f*AUC/MIC: 168
ATCC 27853 and PAO1 control	0	0	0
1	18	36	84
4	4.5	9	21
10	1.8	3.6	8.4
24	0.75	1.5	3.5
PAOΔ*mutS* control	0	0	0
1	36	72	168
4	9	18	42
10	3.6	7.2	16.8
24	1.5	3	7

Overall exposures were achieved by exposing the bacteria to appropriate tobramycin concentrations for the durations of 1, 4, 10 and 24 h, as reported in Table 1. Tobramycin was dosed at 0 h and rapidly removed at the respective timepoint via two or three sequential centrifugation and resuspension steps, as we previously described.[29] This method assured that the tobramycin concentrations were negligible (<0.16× MIC) after drug removal. These different exposures were studied at initial inocula of 10^6 and 10^4 cfu/mL (all three strains), as well as $10^{1.2}$ cfu/mL (PAOΔ*mutS* only). The probability of at least one pre-existing bacterial cell that was resistant to 2.5 mg/L tobramycin was ≤7.1% at 10^4 cfu/mL for ATCC 27853 and PAO1, and 2.3% at $10^{1.2}$ cfu/mL for PAOΔ*mutS*. In contrast, for all strains at 10^6 cfu/mL and PAOΔ*mutS* at 10^4 cfu/mL, this probability was >96.7%. All studies included a growth control. Viability counts of bacteria as described previously[29] were determined within 5 min prior to dosing and at 0.5, 2, 6, 10 (or 12) and 24 h after dosing as well as 5 min before and 10 min after drug removal to confirm minimal loss of bacteria via the drug removal procedure.

Emergence of resistance

The proportion of resistant bacteria (PRB) and MICs were determined at 0 (i.e. before treatment) and 24 h. Tobramycin was removed before evaluating emergence of resistance. Agar dilution MICs were determined once the bacterial suspensions were spectrophotometrically adjusted (i.e. dilution in fresh, pre-warmed, sterile CAMHB) to an inoculum of 10^6 cfu/mL, unless the suspension was already below this inoculum. Agar plates containing 1.25 mg/L (PAO1), 1.5 mg/L (ATCC 27853), 2.5 mg/L (all three strains) and 5 mg/L (PAOΔ*mutS*) tobramycin were used for determining the PRB. Antibiotic-containing agar plates were incubated for 3 days and the $\log_{10}$ PRB was calculated as the difference, on $\log_{10}$ scale, between the viability of resistant bacteria on antibiotic-containing agar plates and total population viability on drug-free plates.

Some of the viable counts at 24 h were too low to quantify colonies on antibiotic-containing agar plates. These bacterial suspensions still

provided information on the upper limit of the $\log_{10}$ PRB (e.g. $\log_{10}$ PRB ≤ -6). To include these data we used the following reporting rules. If the PRB was not quantifiable, but the upper limit was within 1 $\log_{10}$ of the PRB for the growth control, we assumed the PRB was unchanged and used the value of the growth control. If the PRB was not quantifiable and the upper limit was >1 $\log_{10}$ higher than the PRB for the growth control, the PRB of this treatment was reported as missing.

Mechanism-based modelling of bacterial killing and resistance

Mechanism-based pharmacokinetic/pharmacodynamic models were developed to characterize the time-course of bacterial killing and emergence of tobramycin resistance.

Life cycle growth model

The growth and replication of *P. aeruginosa* was described by a life cycle growth model that accounts for the underlying biological processes.[35–37] A diagram of the model structure is shown in Figure 1. Inclusion of three pre-existing bacterial populations (the bacterial populations present prior to treatment)—susceptible, intermediate and resistant—best described the observed data. For each of these populations, the model included two bacterial states: state 1 representing the bacteria preparing for replication and state 2 those immediately before replication;[36–39] e.g. cfu_{S1} denotes the susceptible bacteria in state 1 and cfu_{S2} the susceptible bacteria in state 2.

The total bacterial population (cfu_{all}) was defined as the sum of bacteria in all subpopulations and bacterial states:

$$cfu_{all} = cfu_{S1} + cfu_{S2} + cfu_{I1} + cfu_{I2} + cfu_{R1} + cfu_{R2} \quad (1)$$

where the susceptible population in state 1 (cfu_{S1}) was described by

$$\frac{d(cfu_{S1})}{dt} = ALIVE_S \cdot (REP \cdot k_{21} \cdot cfu_{S2} - k_{12} \cdot cfu_{S1} - KILL_{SPAE1} \cdot cfu_{S1}) \quad (2)$$

and the susceptible population in state 2 (cfu_{S2}) was described by

$$\frac{d(cfu_{S2})}{dt} = ALIVE_S \cdot (-k_{21} \cdot cfu_{S2} + k_{12} \cdot cfu_{S1} - KILL_{SPAE1} \cdot cfu_{S2}) \quad (3)$$

The intermediate (cfu_{I1}, cfu_{I2}) and resistant (cfu_{R1}, cfu_{R2}) populations were modelled similarly.

In Equations (2) and (3), the first-order growth rate constant k_{12} was defined as 60/MGT, with MGT representing the mean generation time for each of the bacterial populations. The first-order replication rate constant k_{21} was set to 50 h^{-1} as described previously.[37] $ALIVE_S$ was 1 while the estimated ($cfu_{S1} + cfu_{S2}$) was $\geq$0.5 cells in the entire broth volume, otherwise $ALIVE_S$ was 0. This part of the model allowed us to describe bacterial eradication. The replication factor REP defines the probability of successful replication, as described previously.[37]

$$REP = 2 \cdot \left(1 - \frac{cfu_{all}}{cfu_{all} + cfu_{max}}\right) \quad (4)$$

REP approaches 2 when the total bacterial count (cfu_{all}) is low, resulting in a 100% probability of successful replication. As cfu_{all} approaches the maximum population size (cfu_{max}), REP approaches 1, which reflects a 50% probability of successful replication and ensures that cfu_{all} does not exceed cfu_{max}. The term $KILL_{SPAE1}$ describes bacterial killing by tobramycin including the post-antibiotic effect (PAE),[40] as described in Equations (5) and (6) below.

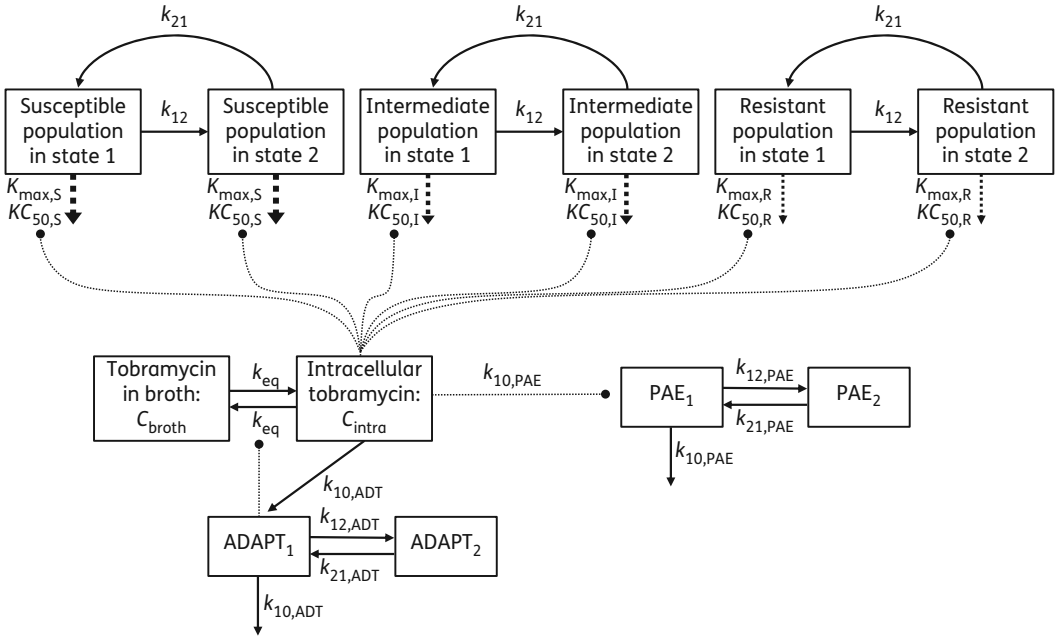

Figure 1. Model diagram of the life cycle growth model including three populations, susceptible, intermediate and resistant, with two states each to describe bacterial replication. The maximum killing rate constants (K_{max}) and the antibiotic concentrations (KC_{50}) causing 50% of K_{max} are explained in Table 2. The concentration of tobramycin in broth (C_{broth}), intracellular tobramycin (C_{intra}), $ADAPT_1$, $ADAPT_2$ and all corresponding rate constants are described in the Materials and methods section.

Bacterial killing by tobramycin including PAE

A prolonged PAE is a trait of tobramycin[40] hence it is represented in the bacterial killing function $KILL_{SPAE1}$ in Equation 5. The $KILL_{SPAE2}$ (Equation 6) is in equilibrium with $KILL_{SPAE1}$ and provides a delay such that bacterial killing occurs for some time after removal of tobramycin from the system.

$$\frac{d(KILL_{SPAE1})}{dt} = k_{10,PAE} \cdot (KILL_S - KILL_{SPAE1}) - k_{12,PAE} \cdot KILL_{SPAE1} + k_{21,PAE} \cdot KILL_{SPAE2} \quad (5)$$

$$\frac{d(KILL_{SPAE2})}{dt} = k_{12,PAE} \cdot KILL_{SPAE1} - k_{21,PAE} \cdot KILL_{SPAE2} \quad (6)$$

The PAE rate constants $k_{10,PAE}$, $k_{12,PAE}$ and $k_{21,PAE}$ represent the delayed killing that results from the PAE and were defined as $k_{10,PAE}=1/MTT_{10,PAE}$, $k_{12,PAE}=1/MTT_{12,PAE}$ and $k_{21,PAE}=1/MTT_{21,PAE}$, where MTT denotes mean turnover time. Modelling the PAO1 did not require this PAE.

The bacterial killing by tobramycin is represented by $KILL_S$:

$$KILL_S = \frac{K_{max,S} \cdot C_{intra}}{C_{intra} + KC_{50,S}} \quad (7)$$

where C_{intra} is the (estimated) intracellular tobramycin concentration. $K_{max,S}$ is the maximum killing rate constant and $KC_{50,S}$ is the C_{intra} required to achieve 50% of $K_{max,S}$.[9] Bacterial killing and PAE of the I and R populations were described similarly. The C_{intra} is defined in Equation (8), as described previously.[9]

$$\frac{d(C_{intra})}{dt} = k_{eq} \cdot C_{broth} - k_{eq} \cdot (ADAPT_1 + 1) \cdot C_{intra} \quad (8)$$

The rate constant k_{eq} is defined as $\ln(2)/t_{1/2eq}$, where $t_{1/2eq}$ is the equilibration half-life between tobramycin in broth and the intracellular space. The term $ADAPT_1$ reflects adaptive resistance of *P. aeruginosa* against tobramycin. An increase in adaptive resistance ($ADAPT_1$, defined below) decreases C_{intra} and thereby reduces the extent of bacterial killing by tobramycin.

Adaptive resistance to tobramycin

Incorporating adaptive resistance in the mechanism-based model was required to describe the observed bacterial count profiles in our experiments. Adaptive resistance against aminoglycosides is often caused by the overexpression of the MexXY-OprM efflux pump[41] or inhibition of energy-dependent uptake in *P. aeruginosa*,[42] both of which would decrease C_{intra}, as described above. It was described by a two-compartment model for $ADAPT_1$ (Equation 9) and $ADAPT_2$ (Equation 10)[9]:

$$\frac{d(ADAPT_1)}{dt} = k_{10,ADT} \cdot \left(\frac{ADAPT_{max} \cdot C_{intra}}{C_{intra} + EC_{50,ADT}} - ADAPT_1\right) - k_{12,ADT} \cdot ADAPT_1 + k_{21,ADT} \cdot ADAPT_2 \quad (9)$$

$$\frac{d(ADAPT_2)}{dt} = k_{12,ADT} \cdot ADAPT_1 - k_{21,ADT} \cdot ADAPT_2 \quad (10)$$

where $ADAPT_{max}$ is the maximum extent of adaptive resistance and $EC_{50,ADT}$ is the C_{intra} that induces the half-maximum extent of adaptive resistance. The peripheral adaptation compartment $ADAPT_2$ allows for the delayed decline of adaptive resistance after removal of tobramycin. The adaptive resistance rate constants $k_{10,ADT}$, $k_{12,ADT}$ and $k_{21,ADT}$ describe the time-course of adaptive resistance and are defined as $k_{10,ADT}=1/MTT_{10,ADT}$, $k_{12,ADT}=1/MTT_{12,ADT}$ and $k_{21,ADT}=1/MTT_{21,ADT}$.

Resistant bacterial populations on antibiotic-containing agar plates

The viable counts on tobramycin-containing agar plates were modelled simultaneously with the total viable counts on drug-free agar plates. The fractions of subpopulations (susceptible, intermediate, resistant) that were able to grow on tobramycin-containing agar plates at different concentrations were estimated as described previously.[43]

Initial conditions

The total initial inocula ($\log_{10}cfu_{0,4}$, $\log_{10}cfu_{0,6}$) and the PRB for the intermediate ($\log_{10}PRB_I$) and resistant ($\log_{10}PRB_R$) populations were estimated (Table 2). The initial condition for the susceptible population was calculated by subtracting the initial conditions of the intermediate and the resistant populations from the respective total inoculum. All bacteria were initialized in state 1 and the initial conditions for cfu_{S2}, cfu_{I2} and cfu_{R2} were set to 0. $ALIVE_S$, $ALIVE_I$ and $ALIVE_R$ were initialized at 1 and all other equations at 0.

Observation model

The $\log_{10}$ viability counts were fitted using an additive residual error model on $\log_{10}$ scale. A previously described residual error model was utilized to fit directly the number of colonies on a plate when there were less than two colonies per plate observed.[44] Viable counts below the limit of counting and model predictions <0 $\log_{10}$ cfu/mL were plotted as 0.

Estimation

The model parameters were estimated simultaneously using the viable counts on drug-free and tobramycin-containing plates for each of the strains at the 10^4 and 10^6 cfu/mL inocula using the importance sampling algorithm (pmethod=4) in parallelized S-ADAPT (version 1.57), facilitated by SADAPT-TRAN.[45] A coefficient of variation of 15% during the end of estimation allowed the between-curve variability of the parameters to be fixed.[44] The objective function, standard diagnostic plots, plausibility of the parameter estimates and visual predictive checks were utilized to assess competing models.

Results and discussion

In the present study, we demonstrated that different shapes of the concentration–time profile had an important impact on bacterial regrowth and emergence of resistance in *P. aeruginosa* ATCC 27853, PAO1 and PAOΔ*mutS*. The $\log_{10}$ PRB for ATCC 27853 was −5.36 on 1.5 mg/L tobramycin plates and −6.43 on 2.5 mg/L tobramycin plates before treatment. PAO1 had a $\log_{10}$ PRB of −5.34 on 1.25 mg/L and −6.77 on 2.5 mg/L tobramycin plates before treatment. The hypermutable PAOΔ*mutS* had a $\log_{10}$ PRB before treatment of −4.14 on 2.5 mg/L and −5.62 on 5 mg/L tobramycin plates. These results show a dramatic difference in PRB before treatment observed between the PAOΔ*mutS* and the two WT strains (ATCC 27853 and PAO1) on 2.5 mg/L tobramycin plates, which was expected due to the hypermutable strain having an increased likelihood of mutating.

The extent of initial killing of *P. aeruginosa* increased with tobramycin exposure (*f*AUC/MIC) for all three strains, as expected for this fast-acting aminoglycoside. At a given *f*AUC/MIC, the extent of initial bacterial killing increased with concentration as the duration of exposure became shorter (Figure 2). In general, at a given *f*AUC/MIC, the high concentrations for short durations

Table 2. Population parameter estimates for tobramycin against three strains of *P. aeruginosa*

Parameter	Symbol (unit)	Population estimate (SE%) for the strain		
		ATCC 27853	PAO1	PAOΔ*mutS*
Bacterial growth and subpopulations				
$\log_{10}$ initial inoculum				
10^4 cfu/mL	$\log_{10}cfu_{0,4}$	4.20 (4.32)	4.23 (1.49)	4.50 (1.97)
10^6 cfu/mL	$\log_{10}cfu_{0,6}$	5.94 (2.84)	5.56 (1.78)	6.05 (1.82)
MGT				
susceptible population	MGT_S (min)	87.3 (7.24)	41.6 (1.86)[a]	63.9 (8.67)
intermediate population	MGT_I (min)	49.3 (3.59)	—	53.5 (3.55)
resistant population	MGT_R (min)	79.9 (8.89)	—	82.2 (6.75)
$\log_{10}$ maximum population size	$\log_{10}cfu_{max}$	9.09 (2.25)	8.95 (1.25)	9.16 (1.66)
$\log_{10}$ PRB				
intermediate population	$\log_{10}PRB_I$	−4.25 (3.47)	−4.58 (2.43)	−4.12 (3.79)
resistant population	$\log_{10}PRB_R$	−7.17 (2.73)	−6.39 (1.11)	−5.60 (4.64)
Bacterial killing by tobramycin				
equilibrium half-life between tobramycin in broth and the intracellular space	$t_{1/2eq}$ (min)	25.0, fixed	40.0 (3.17)	25.0, fixed
maximum killing rate constant				
susceptible population	$K_{max,S}$ (h^{-1})	78.9 (13.6)	30.3 (3.25)	24.8 (9.91)
intermediate population	$K_{max,I}$ (h^{-1})	2.23 (20.9)	2.07 (4.64)	2.02 (13.1)
resistant population	$K_{max,R}$ (h^{-1})	14.2 (12.0)	7.38 (5.10)	7.93 (10.3)
intracellular tobramycin concentration causing 50% of K_{max}				
susceptible population	$KC_{50,S}$ (mg/L)	0.154 (15.4)	0.0206 (15.7)	0.0410 (19.6)
intermediate population	$KC_{50,I}$ (mg/L)	0.0295 (23.9)	0.0270 (31.6)	0.115 (31.9)
resistant population	$KC_{50,R}$ (mg/L)	2.89 (26.4)	0.574 (8.03)	2.79 (15.3)
Adaptive resistance				
maximum extent of stimulation of adaptive resistance	$ADAPT_{max}$	13.5 (20.5)	14.2 (4.49)	11.2 (28.1)
intracellular tobramycin concentration causing 50% of $ADAPT_{max}$	$EC_{50,ADT}$ (mg/L)	6.06 (51.9)	11.1 (4.82)	28.8 (9.33)
MTT				
for adaptive resistance	$MTT_{10,ADT}$ (h)	6.81 (23.2)	7.37 (6.81)	13.5 (9.72)
for distribution from the central to the peripheral adaptive compartment	$MTT_{12,ADT}$ (h)	0.917 (50.6)	0.972 (10.3)	0.439 (32.2)
for distribution from the peripheral to the central adaptive compartment	$MTT_{21,ADT}$ (h)	6.0, fixed	6.0, fixed	6.0, fixed
PAE				
MTT				
for the PAE	$MTT_{10,PAE}$ (h)	0.0166 (56.9)	—	0.0158 (29.2)
between the central and peripheral PAE compartment	$MTT_{12,PAE}$ (h)	0.519 (43.3)	—	1.06 (22.0)
between the peripheral and central PAE compartment	$MTT_{21,PAE}$ (h)	0.662 (16.2)	—	0.504 (12.6)
Residual variability				
SD of additive residual error on $\log_{10}$ scale for the				
total population	SD_{cfu}	0.302	0.455	0.459
population on 2.5 mg/L tobramycin plates	SD_{cfu3}	0.137	1.51	0.195
population on 5 mg/L tobramycin plates	SD_{cfu5}	0.307	1.79	0.399

[a]Model only contains one MGT.

of exposure resulted in more extensive regrowth than exposure to lower concentrations over a longer time (Figure 2). It is of great concern that for all strains even the high *f*AUC/MIC of 168, i.e. four times the suggested *f*AUC/MIC breakpoint of 42 for bactericidal effect,[24] did not inhibit the regrowth of bacteria at the initial inoculum of 10^6 cfu/mL. An *f*AUC/MIC of 168 would be expected to be achieved in patients for MICs up to ∼0.5 mg/L following a tobramycin dose of 5–6 mg/kg in critically and non-critically ill patients[32,46] and 8–11 mg/kg in patients with CF (both based on a 70 kg patient).[47,48] However, according to EUCAST, 46% of the 25002 evaluated isolates had an MIC ≥1 mg/L.[31]

Generally, with short durations of exposure (1 and 4 h) the ATCC 27853 strain displayed rapid initial killing of 4–6 $\log_{10}$ that was followed by extensive regrowth after drug removal, whilst the longer durations of exposure (10 and 24 h) had limited or no regrowth (Figure 2a). An exception was the low *f*AUC/MIC of 36 where the longer durations of exposure revealed 4 $\log_{10}$ regrowth. In addition, at the high *f*AUC/MIC of 168 the 4 h

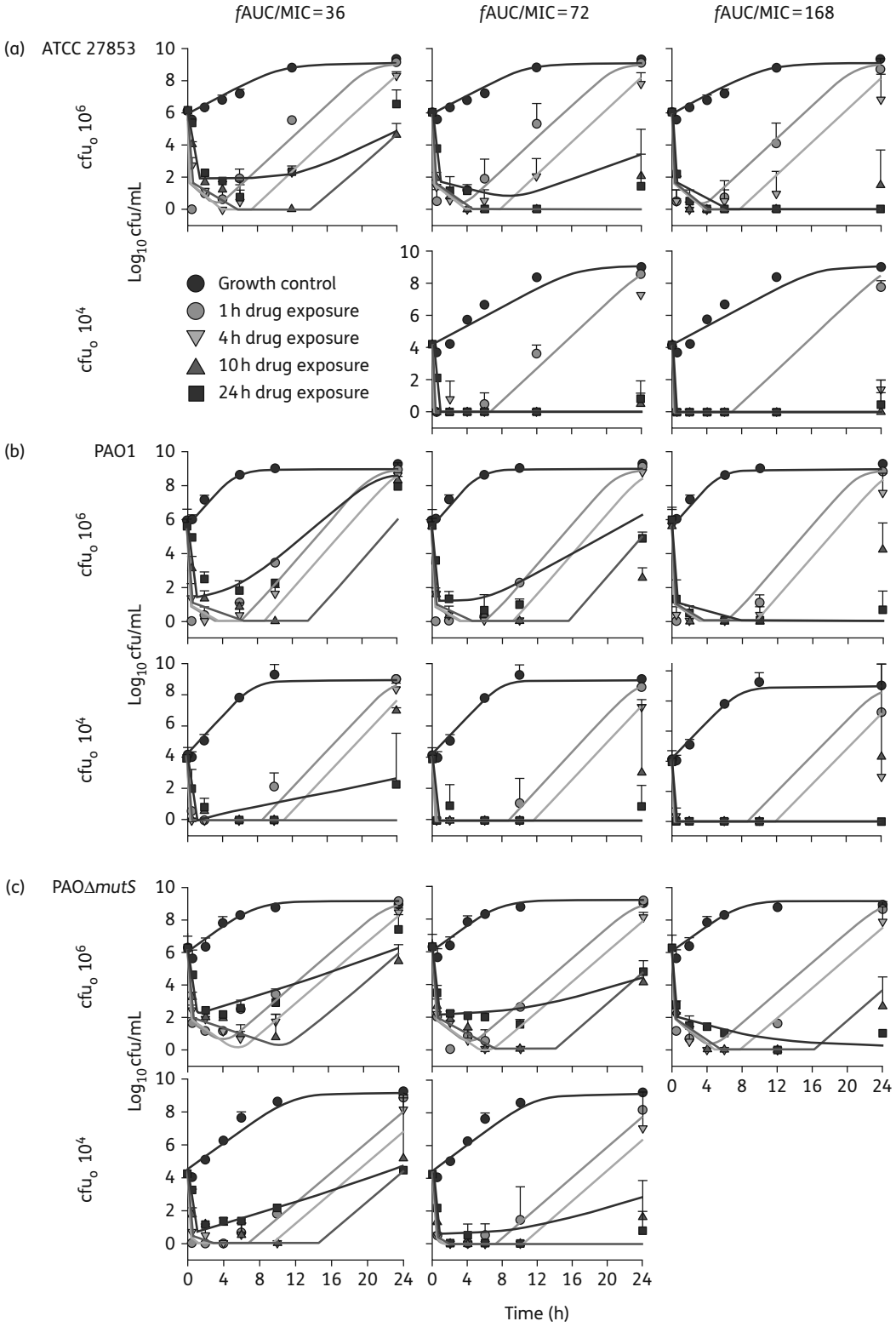

Figure 2. Observed viable counts (mean ± SD) and population predicted profiles (continuous lines in corresponding colours) for *P. aeruginosa* ATCC 27853 (a), PAO1 (b) and PAOΔ*mutS* (c) exposed to tobramycin at an *f*AUC/MIC of 36 (left column), 72 (middle column) and 168 (right column) delivered over 1, 4, 10 or 24 h durations of exposure, at initial inocula (cfu$_o$) of 10^6 and 10^4 cfu/mL, excluding *f*AUC/MIC of 36 for ATCC 27853 and *f*AUC/MIC of 168 for PAOΔ*mutS* for cfu$_o$ 10^4 cfu/mL. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

duration of exposure showed limited regrowth at the 10^4 cfu/mL initial inoculum that likely lacked pre-existing resistant bacteria (Figure 2a). The PAO1 strain showed similar results to the ATCC 27853 strain, with similar extents of initial killing, and mostly the shorter durations of exposure leading to extensive regrowth (Figure 2b). However, the PAO1 strain showed more extensive regrowth for the longer durations of exposure at an *f*AUC/MIC of 36 in comparison with ATCC 27853; extensive regrowth was observed for the 10 and 24 h durations of exposure for PAO1 at the 10^6 cfu/mL inoculum (Figure 2b). The initial killing and bacterial regrowth of PAOΔ*mutS* was mostly comparable to its parental strain PAO1 and ATCC 27853 (Figure 2). At the very low initial inoculum of $10^{1.2}$ cfu/mL complete eradication at 24 h was observed for all tobramycin exposures against PAOΔ*mutS*, except for the 1 h duration of exposure at *f*AUC/MIC of 36 where there was ~2 $\log_{10}$ regrowth (data not shown).

Bacterial regrowth was a common feature of the time–kill profiles for each strain (Figure 2). Notably, at the 10^6 cfu/mL inoculum, although the shorter durations of exposure (1 and 4 h) had more extensive regrowth, their PRB at 24 h were comparable to the growth control for all three strains, suggesting that this regrowth was a susceptible population (Figure 3). Consideration should be given to combination antibiotic therapy as an option for fighting this susceptible regrowth, i.e. using a second antibiotic to prevent the regrowth of the tobramycin-susceptible bacteria. In contrast, the longer durations of exposure (10 and 24 h) at the 10^6 cfu/mL inoculum frequently resulted in increased PRB at 24 h for all three strains at an *f*AUC/MIC of 36 and 72 (Figure 3). These increases in PRB were mostly supported by raised MIC at 24 h for the 10 and 24 h durations of exposure (Table 3). Overall, the hypermutable PAOΔ*mutS* strain had considerably higher PRB values compared with the two WT strains (Figure 3).

For ATCC 27853 and PAO1 the increase in PRB at longer durations could be prevented or considerably limited by the high *f*AUC/MIC of 168 at the 10^6 cfu/mL inoculum, except for the PAO1 at the 24 h duration of exposure. In contrast, for PAOΔ*mutS* essentially the whole bacterial population was replaced by resistant bacteria even at the *f*AUC/MIC of 168. This difference was observed despite the same probability of >96.7% of at least one pre-existing resistant bacterial cell for all three strains. Thus our study demonstrates that resistance suppression is more challenging for the hypermutable strain compared with the two non-hypermutable strains, which is supported by literature reporting that hypermutation results in more difficult-to-eradicate infections.[17,21]

For the 10^4 cfu/mL inoculum, ATCC 27853 and PAO1 exposed to tobramycin for short durations (1 and 4 h) had limited to no increase in PRB and for ATCC 27853 the PRB was unquantifiable for long durations of exposure (Figure 3). These results were supported by the MIC at 24 h remaining unchanged for the 1 and 4 h durations of exposure (Table 3). However, for PAO1 the PRB slightly increased for the 10 and 24 h durations of exposure for the low *f*AUC/MIC of 36 (Figure 3); the 10 h duration of exposure was supported by a raised MIC (Table 3). Similarly, the PAOΔ*mutS* at 10^4 cfu/mL inoculum revealed increases in PRB for the 10 h duration of exposure at an *f*AUC/MIC of 36 and the 24 h duration of exposure at an *f*AUC/MIC of both 36 and 72 (Figure 3). The low inoculum of $10^{1.2}$ cfu/mL (~300 bacteria in 20 mL) was required to minimize the probability of pre-existing resistant bacteria for the hypermutable PAOΔ*mutS* strain. No PRB could be determined for this inoculum due to extensive bacterial killing and the very low numbers of bacteria present (data not shown). Although adaptive resistance mechanisms only require tobramycin exposure[49] these may have been prevented by the extremely fast bacterial killing seen at the $10^{1.2}$ cfu/mL inoculum.

The pre-existing resistant bacteria in the initial inoculum may have been playing a role in the emergence of resistance at the 10^6 cfu/mL inoculum for all strains and at 10^4 cfu/mL for PAOΔ*mutS*. Treatment failure in *P. aeruginosa* infections often occurs from selection of resistant bacteria, e.g. due to overexpression of the MexXY-OprM efflux pump, aminoglycoside-modifying enzymes and decreased outer membrane permeability.[11–14] For PAO1 it is likely that additional resistance pathways not involving amplification of pre-existing resistant bacteria may have played a role in resistance emergence at the 10^4 cfu/mL inoculum. The static concentrations with complete removal of tobramycin at the end of each exposure period would have prevented the development of resistance mechanisms that require sub-MIC concentrations to occur. Common mechanisms involved in the resistance observed in PAO1 could be adaptive resistance via the upregulation of the MexXY-OprM efflux pump,[41,50] which is frequently caused by overexpression of the MexY component, or *de novo* mutations during treatment.[41,51,52] Previous studies have found that a resistance mechanism unrelated to this efflux pump has played a role in aminoglycoside resistance for PAOΔ*mutS*.[6] Ultimately, molecular studies would identify these resistance mechanisms.

Our developed mechanism-based model successfully described simultaneously the time-course of viable counts for the total bacterial population and less susceptible bacterial populations growing on tobramycin-containing plates, for all three strains (Figure 2 and Figure S1, available as Supplementary data at *JAC* Online). The model included three bacterial subpopulations, i.e. susceptible, intermediate and resistant (Figure 1). Tobramycin causes bacterial killing via inhibiting protein synthesis,[8] which is driven by the intracellular tobramycin concentration as described in the model.[9] Aminoglycosides can also cause bacterial killing by disrupting the outer membrane;[10,53,54] however, adding a second mechanism of killing was not required to describe our observed data. It has been suggested that the cellular recovery after tobramycin exposure causes a delay in bacterial regrowth, resulting in a PAE that was included in the model.[40,55] Such a PAE may allow the suppression of regrowth of susceptible subpopulations in patients.[56] The PAE duration has been found to be related to the antibacterial concentration,[57,58] the duration of drug exposure and the inoculum size.[59] A longer duration of exposure was found to result in a longer PAE.[40] The ATCC 27853 and PAOΔ*mutS* strains required a PAE to be present in the model, whilst PAO1 was represented by a simplified model that did not require a PAE.

The bacterial regrowth was also well captured by the model with only minor mispredictions of up to ~2.5 $\log_{10}$. Only for 2 of 80 modelled profiles (ATCC 27853 at the *f*AUC/MIC of 72, 4 h duration of exposure and PAO1 at the *f*AUC/MIC of 36, 10 h duration of exposure, 10^4 cfu/mL inoculum) a larger misprediction occurred as the model predicted complete eradication before bacteria were able to regrow (Figure 2). Emergence of resistance as quantified via tobramycin-containing agar plates was adequately represented in the model (Figure S1). The inclusion of adaptive resistance in the model allowed us to consider the likely cause of emergence of aminoglycoside resistance at low initial inocula.[42,60–63] Incorporating a function for adaptive resistance in

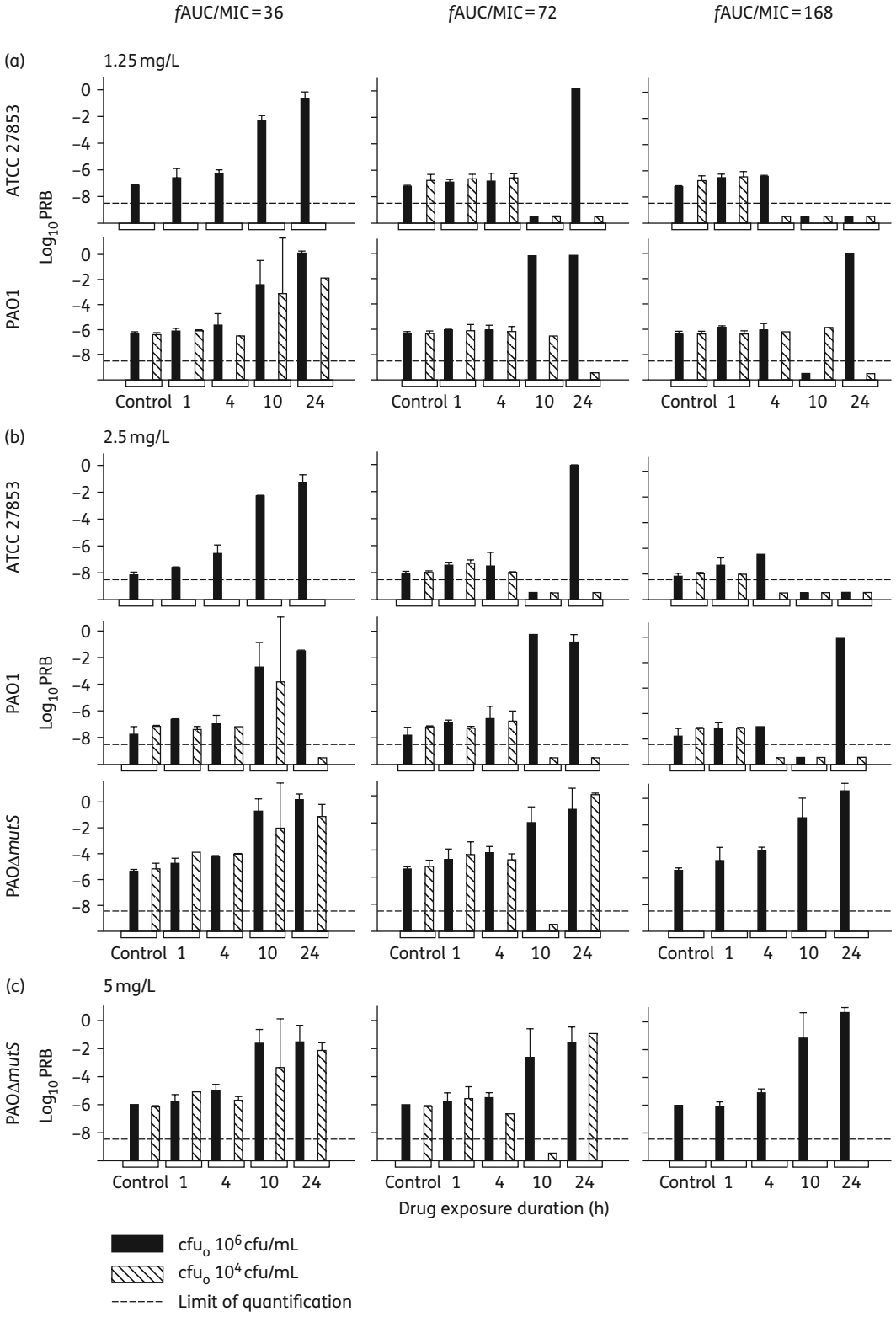

Figure 3. Log_{10} PRB (mean ± SD) at 24 h on agar plates containing 1.25 (1.5 for ATCC 27853; a), 2.5 (b) and 5 (c) mg/L tobramycin. This figure shows *f*AUC/MIC values of 36 (left column), 72 (middle column) and 168 (right column) for the *P. aeruginosa* ATCC 27853, PAO1 and PAOΔ*mutS* delivered over 1, 4, 10 or 24 h durations of exposure, at initial inocula (cfu_o) of 10^6 and 10^4 cfu/mL, excluding *f*AUC/MIC of 36 for ATCC 27853 and *f*AUC/MIC of 168 for PAOΔ*mutS* for cfu_o 10^4 cfu/mL. When a treatment yielded complete eradication or when there were no colonies on antibiotic-containing agar plates no PRB could be determined, and therefore is shown below the limit of quantification as -9.5 log_{10}.

Table 3. MIC (mg/L) at 24 h [geometric mean (range)] for tobramycin *f*AUC/MIC of 36, 72 and 168 delivered over various durations of exposure and initial inocula (cfu_o) against *P. aeruginosa* ATCC 27853 (top), PAO1 (middle) and PAOΔ*mutS* (bottom)

	*f*AUC/MIC: 36		*f*AUC/MIC: 72		*f*AUC/MIC: 168	
Drug exposure duration (h)	cfu_o 1×10^6	cfu_o 1×10^4	cfu_o 1×10^6	cfu_o 1×10^4	cfu_o 1×10^6	cfu_o 1×10^4
ATCC 27853 control	0.5 (0.5–0.5)	ND	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.5 (0.5–0.5)
1	0.5 (0.5–0.5)	ND	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.5 (0.5–0.5)
4	0.5 (0.5–0.5)	ND	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.25
10	1.0 (0.5–2.0)	ND	0.5	—	0.25	—
24	**4.0 (4.0–4.0)**	ND	**4.0**	—	—	—
PAO1 control	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.5 (0.5–0.5)
1	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.5 (0.5–0.5)	0.7 (0.5–1.0)	0.7 (0.5–1.0)	0.5 (0.5–0.5)
4	0.7 (0.5–1.0)	0.5 (0.5–0.5)	0.7 (0.5–1.0)	0.7 (0.5–1.0)	0.7 (0.5–1.0)	0.5
10	**2.0 (0.5–8.0)**	**2.0 (0.5–8.0)**	0.25	1.0	0.5 (0.25–1.0)	—
24	**5.7 (4.0–8.0)**	—	**4.0 (4–4)**	—	—	—
PAOΔ*mutS* control	1.0 (1.0–1.0)	1.0 (1.0–1.0)	1.0 (1.0–1.0)	1.0 (1.0–1.0)	1.0 (1.0–1.0)	ND
1	1.0 (1.0–1.0)	2.8 (2.0–4.0)	1.0 (1.0–1.0)	2.0 (2.0–2.0)	1.0 (1.0–1.0)	ND
4	1.0 (1.0–1.0)	2.0 (2.0–2.0)	1.0 (1.0–1.0)	1.0 (1.0–1.0)	1.0 (1.0–1.0)	ND
10	**8.0 (8.0–8.0)**	1.0	0.7 (0.5–1.0)	—	—	ND
24	**11.3 (8.0–16.0)**	**5.7 (4.0–8.0)**	**5.7 (4.0–8.0)**	—	—	ND

ND indicates that this inoculum was not carried out for this strain in this study.
MICs are in bold if they were at least 4-fold above baseline. No range is provided if only one replicate was available. PAOΔ*mutS* at cfu_o of $10^{1.2}$ cfu/mL had MIC 1.0 mg/L with a range 1.0–1.0 mg/L for the growth control, whilst extensive bacterial killing did not allow determination of the MIC for any treated arms.

addition to amplification of pre-existing resistant bacteria was required to describe best the observed data. Overall, the model accurately described the bacterial killing (Figure 2) and emergence of resistance (Figure S1) for all three strains. We recognize that both the *in vitro* experiments and mechanism-based model lack an immune system effect and therefore the results of this study would be most applicable to immunocompromised patients. In addition, the static nature of the concentration delivery for defined durations generated different concentration–time profiles to those that would be observed in patients.

In conclusion, the study allowed us to determine whether different tobramycin concentration–time profiles at a given overall exposure affect not only bacterial killing, but also resistance prevention. Our results for the 24 h duration of exposure demonstrated that, despite limited regrowth, there was complete replacement of susceptible bacteria with tobramycin-resistant populations when regrowth occurred. Emergence of resistance was suppressed for 1 and 4 h durations of exposure supporting once-daily dosing, although extensive regrowth of susceptible bacteria occurred. Therefore, investigation of dosage regimens involving short-duration, high tobramycin concentrations together with a second antibiotic to prevent tobramycin-susceptible regrowth is warranted. Our mechanism-based mathematical model would assist in the optimization of such antibiotic combinations. Combination dosage regimens may be particularly beneficial and are urgently required to combat hypermutable strains arising in patients. Studies in dynamic *in vitro* systems that simulate antibiotic concentration–time profiles as observed in patients are necessary to evaluate such innovative aminoglycoside combination dosage regimens and translate these regimens to benefit patients.

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Transparency declarations

None to declare.

Disclaimer

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Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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Supplementary material

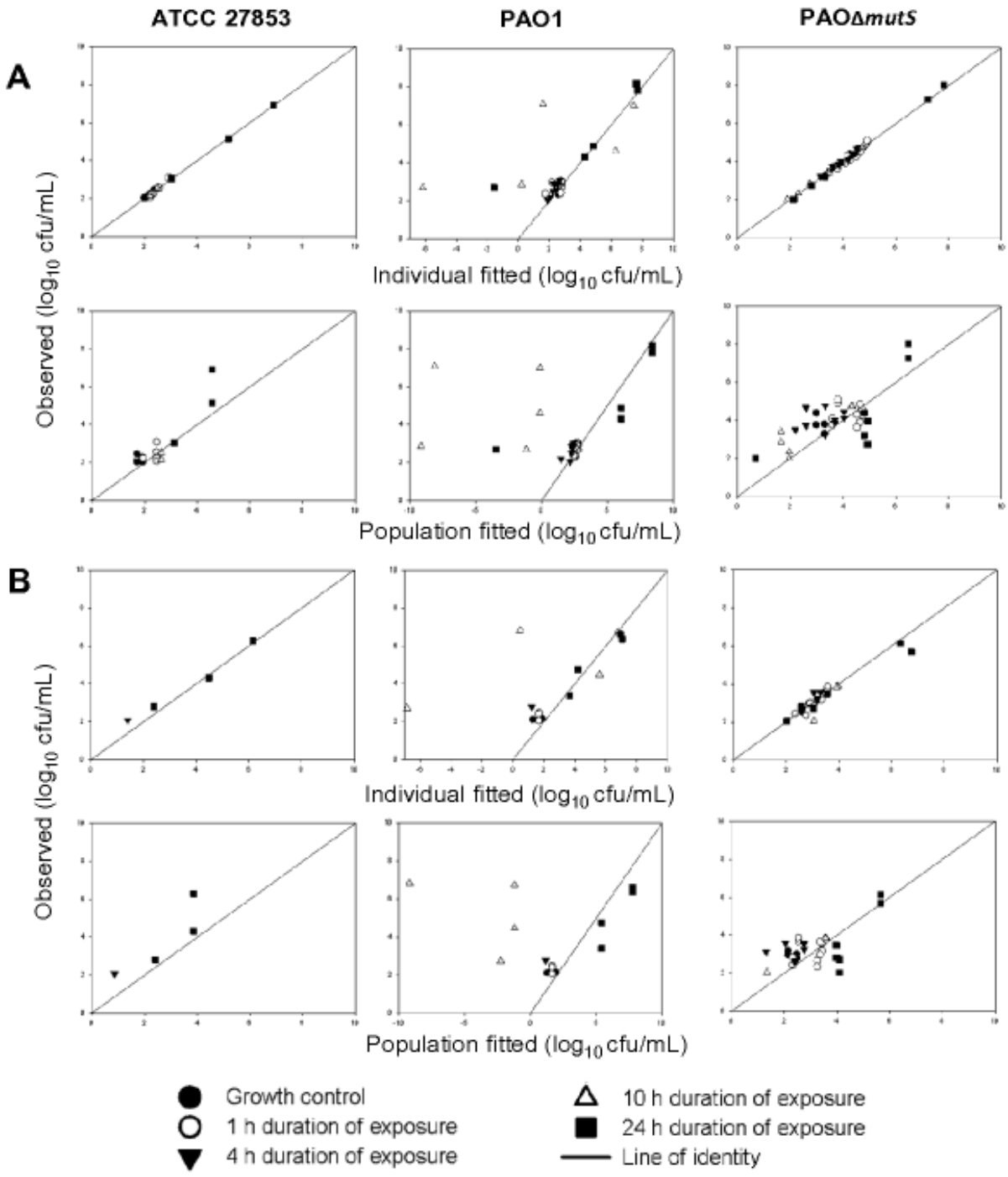

Figure S1: Observed versus individual (top) and population fitted (bottom) viable counts for both 1.25 mg/L for PAO1, 1.5 mg/L for ATCC 27853 and 2.5mg/L for PAOΔ*mutS* (**A**); 2.5 mg/L for PAO1 and ATCC 278853, and 5mg/L for PAOΔ*mutS* (**B**).

Chapter 4

Impact of hypermutation on the emergence of resistance of *Pseudomonas aeruginosa* exposed to clinically relevant concentrations of diverse antibiotics

Preamble

MICs of available antibiotics against hypermutable and non-hypermutable *P. aeruginosa* have shown that hypermutation leads to much higher emergence of resistance. However, the impact of hypermutation on emergence of resistance had not been systematically evaluated *via* static concentration time-kill assays for a wide range of antibiotics from different classes at clinically representative inocula. This Chapter addresses **Aim 2** through the investigation of the antibacterial effects of clinically important antibiotics in monotherapy against isogenic hypermutable and non-hypermutable *P. aeruginosa* laboratory strains in 48-h static concentration time-kill experiments. The results support **Hypothesis 2** as greater resistance emergence was observed for hypermutable compared to non-hypermutable *P. aeruginosa* against aztreonam, ceftazidime, imipenem, meropenem, tobramycin and ciprofloxacin. This Chapter further addressed **Aim 2** by comprehensively investigating synergy and resistance suppression by a combination of two fast-acting antibiotics against these isogenic, hypermutable and non-hypermutable *P. aeruginosa* laboratory strains. Due to the regrowth of susceptible populations observed against ciprofloxacin in Chapter 2 and tobramycin in Chapter 3, and their different mechanisms of action and of resistance, the combination of these antibiotics was tested. This synergistic tobramycin plus ciprofloxacin combination showed that combinations of available antibiotics can maximise bacterial killing and minimise resistance emergence of hypermutable *P. aeruginosa*.

Submitted manuscript

Impact of hypermutation on the emergence of resistance of *Pseudomonas aeruginosa* exposed to clinically relevant concentrations of diverse antibiotics

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Abstract

Hypermutable *Pseudomonas aeruginosa* strains have a greatly increased mutation rate and are prevalent in chronic respiratory infections. These strains have been associated with enhanced emergence of resistance, creating a dire need to improve the efficacy of antibiotic therapy and to suppress resistance. This study aimed to systematically evaluate the bacterial time-course of the total and resistant populations of hypermutable (PAOΔ*mutS*) and non-hypermutable (PAO1) *P. aeruginosa* strains exposed to clinically important antibiotics (aztreonam, ceftazidime, imipenem, meropenem, tobramycin and ciprofloxacin). Both strains were examined in static concentration time-kill studies at two inocula to assess bacterial killing and emergence of resistant populations. All antibiotics were evaluated in monotherapy. In addition, the combination of tobramycin and ciprofloxacin was studied. Serial viable counts of the total and resistant populations were determined. With monotherapy, bacterial regrowth at 48 h was generally more pronounced for PAOΔ*mutS* compared to PAO1. Furthermore, PAOΔ*mutS* demonstrated greater emergence of resistant populations overall than PAO1 for all tested antipseudomonal antibiotics in monotherapy. The combination of tobramycin and ciprofloxacin was synergistic with up to $10^{6.1}$ CFU/mL more bacterial killing than the most active monotherapy for both strains. Tobramycin at 1, 4 and 8 mg/L combined with 1 and 4 mg/L ciprofloxacin largely suppressed less-susceptible populations. This work indicated that the use of monotherapy against hypermutable *P. aeruginosa* strains is not a viable option. Tobramycin in combination with ciprofloxacin was identified as a promising and tangible option to combat hypermutable *P. aeruginosa* strains and suppress resistance.

1. Introduction

Respiratory infections caused by *Pseudomonas aeruginosa* are a major cause of morbidity and mortality among patients with cystic fibrosis (CF) [1]. Furthermore, hypermutable *P. aeruginosa* strains are prevalent in chronic respiratory infections of CF patients [2, 3]. These hypermutable strains often result from altered or defective genes within the mismatch repair (MMR) system, which lead to an increased mutation rate [4]. As a result, hypermutable bacteria can quickly adapt to changing environments, including antibiotic exposure [3]. Treatment of *P. aeruginosa* infections involving these hypermutable strains risks the emergence of multi-drug resistance; therefore there is an urgent need to evaluate antibiotic therapy to improve efficacy, including suppression of the emergence of resistance [5].

Previously MIC values have been determined for multiple antibiotics against a hypermutable strain [6, 7]. However, MICs are based on only one time-point, and use a low bacterial inoculum and a small volume of bacterial suspension. Static concentration time-kill assays allow a quantitative description of the time-course of antibacterial effects on the total and resistant bacterial populations. The impact of increased spontaneous mutation rates found in hypermutable *P. aeruginosa* on the time-course of bacterial killing, regrowth and resistance emergence over 48 h has not been systematically evaluated for a range of antibiotics from different classes. Hence, we aimed to quantify the differences in antibacterial effects and emergence of resistant populations between hypermutable and non-hypermutable *P. aeruginosa* for the most important clinically used antipseudomonal antibiotics. Furthermore, we aimed to assess the synergistic properties and suppression of resistant populations by the combination of two fast-acting antibiotics, tobramycin and ciprofloxacin, against hypermutable and non-hypermutable *P. aeruginosa*.

2. Materials and methods

2.1 Bacterial strains and antibiotics tested

We used a well-characterised hypermutable PAOΔ*mutS* strain [8] and its non-hypermutable PAO1 wild-type parental strain [9] (*i.e.* that differ only in the knockout of the *mutS* gene from the MMR system), in 48-h static concentration time-kill studies. Bacterial killing and emergence of resistant populations were quantified for six common antipseudomonals, as follows: aztreonam (Sigma-Aldrich, Castle Hill, Australia), ceftazidime (Chem-Impex, Wood Dale, USA), imipenem (MSD, Macquarie Park, Australia), meropenem (DBL Hospira, Melbourne, Australia), tobramycin (AK Scientific, Union City, USA) and ciprofloxacin (Waterstone Technology, Carmel, USA). All antibiotic solutions were prepared in MilliQ® water and were filter-sterilised using a 0.22 μm PVDF syringe filter (Merck Millipore, Cork, Ireland). The agar MIC values using the CLSI method for both strains were 4 mg/L for aztreonam, 2 mg/L for ceftazidime, 2 mg/L for imipenem, 1 mg/L for meropenem, 0.5 mg/L for tobramycin and 0.125 mg/L for ciprofloxacin.

2.2 Static concentration time-kill assays

Inocula of approximately $10^{5.2}$ and $10^{7.5}$ colony forming units (CFU)/mL for monotherapy and approximately $10^{7.5}$ CFU/mL for combination studies were targeted. Targeted inocula were achieved in 15 mL cation-adjusted Mueller Hinton broth (CAMHB; containing 25 mg/L Ca^{2+} and 12.5 mg/L Mg^{2+}; BD, North Ryde, Australia), and static concentration time-kill studies were conducted for 48 h as described previously [10]. In monotherapy we studied: aztreonam 1, 2, 4, 8, 16, 32, 64 and 128 mg/L; ceftazidime 1, 2, 4, 8, 32 and 64 mg/L; imipenem 0.5, 1, 2, 4, 16 and 64 mg/L; meropenem 0.25, 0.5, 1, 2, 8 and 32 mg/L; tobramycin 0.25, 0.5, 1, 2, 4, 8, 16 and

32 mg/L; and ciprofloxacin 0.0625, 0.125, 0.25, 0.5, 1, 2 and 4 mg/L. At 24 h, 100% re-dosing after a centrifugation and re-suspension step was performed for aztreonam, imipenem and meropenem; the carbapenems also had an additional 30% (meropenem) or 50% (imipenem) supplementation at 6 and 30 h to offset thermal degradation [11, 12]. The effect of combining two rapidly killing antibiotics was examined using tobramycin (1, 4 and 8 mg/L) and ciprofloxacin (0.125, 1 and 4 mg/L) in mono- and combination therapy.

2.3 Viable counting of the total and resistant populations

Viability counts were determined at 0 (5 min prior to dosing), 1.5, 3, 6, 24, 29 (excluding aztreonam $10^{7.5}$ CFU/mL and ceftazidime at both inocula) and 48 h as previously described [10] to establish the time-course of the total population on drug-free cation-adjusted Mueller Hinton agar (CAMHA; containing 25 mg/L Ca^{2+} and 12.5 mg/L Mg^{2+}; BD, North Ryde, Australia) and resistant populations at 24 and 48 h on antibiotic-containing CAMHA. Antibiotic resistant populations were based on MIC and breakpoint concentrations. The antibiotic concentrations used in CAMHA to quantify the resistant populations for the monotherapy studies were: 20 mg/L aztreonam, 10 mg/L ceftazidime, 10 mg/L imipenem, 5 mg/L meropenem, 2.5 mg/L tobramycin and 1.25 mg/L ciprofloxacin. The tobramycin with ciprofloxacin combination studies examined the less-susceptible populations on 2.5 mg/L tobramycin- and 0.625 mg/L ciprofloxacin-containing CAMHA (a lower ciprofloxacin concentration was used as the combination was anticipated to reduce the emergence of resistant populations). The limit of counting was 1.0 $\log_{10}$ CFU/mL on antibiotic-free agar plates and 0.7 $\log_{10}$ CFU/mL on antibiotic-containing agar plates. Samples with no detectable colonies were plotted at 0 $\log_{10}$ CFU/mL.

3. Results and Discussion

3.1. Antibacterial effect of common antipseudomonal antibiotics in monotherapy

The time-course profiles of bacterial density as determined on antibiotic-free plates (*i.e.* total bacterial count) and antibiotic-containing plates (*i.e.* resistant subpopulations) for the monotherapy investigation are presented in **Fig. 1** and **2**, respectively. The resistant populations of the growth controls (*i.e.* absence of antibiotic during the time-course studies) of hypermutable PAOΔ*mutS* and non-hypermutable PAO1 were generally correlated with their respective spontaneous mutation rates (**Fig. 2**). In the presence of antibiotic during the time-course studies, overall there was less bacterial killing and suppression of resistant populations of PAOΔ*mutS* compared to the PAO1 strain. The collection of multiple samples over 48 h in the present study allowed us to evaluate the antibacterial effects on not only killing but also regrowth and emergence of resistant populations. Our two studied inocula were intended to simulate infections with different bacterial densities.

3.1.1. Antibacterial effect of beta-lactam antibiotics in monotherapy

The β-lactam aztreonam showed differences in bacterial regrowth (**Fig. 1**) and emergence of resistant populations (**Fig. 2**) between the two strains at the lower inoculum. Resistant bacteria had completely replaced the susceptible bacteria by 48 h when PAOΔ*mutS* was exposed to 16 mg/L aztreonam. Similarly, for the lower inoculum with exposure to 32 mg/L aztreonam (equivalent to the unbound average steady-state plasma concentration for the maximum daily dose [13]), almost the whole population of PAOΔ*mutS* at 48 h (~5.7 $\log_{10}$ CFU/mL) was replaced by resistant bacteria. There was very limited antibacterial effect at the higher inoculum of both strains. However, PAO1 showed a reduced extent of aztreonam-resistant populations compared to

PAOΔ*mutS*. The β-lactam ceftazidime against the lower inoculum had earlier bacterial regrowth of PAOΔ*mutS* compared to PAO1 (**Fig. 1**). Furthermore, there was much less antibacterial effect for both strains at the higher than at the lower inoculum. While the size of the resistant population at 48 h was comparable between the strains at both inocula, higher resistant bacterial counts were generally observed for the PAOΔ*mutS* compared to PAO1 at 24 h (**Fig. 2**). For both ceftazidime and aztreonam a pronounced inoculum effect was observed for both strains (**Fig. 1**), as described previously for non-hypermutable *P. aeruginosa* [14, 15].

The carbapenem imipenem had mostly comparable antibacterial effects between the strains at both inocula (**Fig. 1** and **2**). However, a 4× higher imipenem concentration (64 mg/L that is not clinically achievable *vs.* clinically achievable 16 mg/L [13]) was required to largely suppress regrowth and resistant populations of PAOΔ*mutS* compared to PAO1. At the lower inoculum a resistant population of ~6.4 $\log_{10}$ CFU/mL for PAOΔ*mutS*, and none for PAO1, was found at 48 h against the 16 mg/L imipenem (equivalent to the unbound average steady-state plasma concentration for the maximum daily dose of 4 g [13]). Furthermore, the higher inoculum against the 16 mg/L imipenem had a resistant population of PAOΔ*mutS* that was ~4.5 $\log_{10}$ CFU/mL (~34,000-fold) greater than that of PAO1. Imipenem was previously assessed against a very low inoculum (~$10^{4.6}$ $\log_{10}$ CFU/well) of PAO1 and PAOΔ*mutS* in 24-h static concentration time-kill assays at only two concentrations (4 and 8 mg/L) [7]; antibacterial effects were more pronounced for PAO1 than PAOΔ*mutS*.

Exposure to the carbapenem meropenem resulted in substantially greater bacterial regrowth and the emergence of less-susceptible populations for PAOΔ*mutS* compared to PAO1 (**Fig. 1** and **2**). Notably, the higher inoculum against 8 mg/L meropenem (equivalent to the average steady-state plasma concentration in patients receiving the standard daily dose of 3 g

[13]) led to regrowth of PAOΔ*mutS* to ~8.5 $\log_{10}$ CFU/mL at 48 h with almost the whole population (~8.4 $\log_{10}$ CFU/mL) replaced by less-susceptible bacteria. In contrast, regrowth and less-susceptible populations were largely suppressed for PAO1. Interestingly, a previous study assessed intermittent meropenem (1 g thrice daily as 30 min infusions) against PAO1 and a hypermutable clinical *P. aeruginosa* strain (from a wound swab of an intensive care unit patient) in a 24-h dynamic *in vitro* model; this study showed less-susceptible populations for both strains even at 24 h [16].

3.1.2. Antibacterial effect of fast-acting antipseudomonal antibiotics in monotherapy

The aminoglycoside tobramycin initially achieved substantial bacterial killing of both strains (**Fig. 1**). This was followed by more extensive regrowth of less-susceptible populations at 48 h for PAOΔ*mutS* compared to PAO1 at both inocula (**Fig. 2**). At the lower inoculum, a 4× higher concentration (4 *vs.* 1 mg/L) was required to suppress regrowth to <4.0 $\log_{10}$ CFU/mL of PAOΔ*mutS* compared to PAO1. For the high inoculum, tobramycin 8 mg/L suppressed regrowth and less-susceptible populations of PAO1 over 48 h; PAOΔ*mutS* regrew to ~9.2 $\log_{10}$ CFU/mL at 48 h with ~9.1 $\log_{10}$ CFU/mL of less-susceptible bacteria. Notably, 32 mg/L tobramycin (which clinically can only be achieved for a very short time as a peak concentration [13]) was required to suppress regrowth of PAOΔ*mutS*. Previously, we studied tobramycin in 24-h static concentration time-kill studies against both strains at inocula of 10^6 and 10^4 CFU/mL [17]; the results for the 10^6 CFU/mL inoculum in that study were in accord with those for the lower ($10^{5.2}$ CFU/mL) inoculum in the present study.

The fluoroquinolone ciprofloxacin displayed earlier regrowth of PAOΔ*mutS* than PAO1 at both inocula (**Fig. 1**). Ciprofloxacin 4 mg/L (which is not clinically achievable as an unbound

concentration in plasma [13]) at both inocula was required to largely suppress regrowth of PAOΔ*mutS*, whereas 1 mg/L was sufficient for PAO1. This was in agreement with a previous study using static concentration time-kill assays over 24 h where 1 mg/L ciprofloxacin against a low inoculum prevented regrowth of PAO1 whilst PAOΔ*mutS* exhibited regrowth after 6 h [7]. In the present study at 48 h, the lower inoculum against 1 mg/L ciprofloxacin had a resistant population of ~5.9 $\log_{10}$ CFU/mL for PAOΔ*mutS*, and none for PAO1 (**Fig. 2**). Additionally, the higher inoculum against 1 mg/L ciprofloxacin showed the resistant population to be ~3.5 $\log_{10}$ CFU/mL (~3,500-fold) greater for PAOΔ*mutS* than PAO1. A resistant population of ~8.6 $\log_{10}$ CFU/mL for PAOΔ*mutS*, and none for PAO1, was found at the higher inoculum against 2 mg/L ciprofloxacin at 48 h.

For ciprofloxacin and tobramycin, which are clinically only administered intermittently, concentrations at or above the highest clinically achievable unbound peak plasma concentrations were required to suppress regrowth of hypermutable PAOΔ*mutS* (whilst non-hypermutable PAO1 only needed lower clinically achievable concentrations) over 48 h at inocula of $10^{5.2}$-$10^{7.5}$ CFU/mL. This suggests ciprofloxacin and tobramycin in monotherapy would not be expected to be successful against hypermutable *P. aeruginosa* strains.

3.2. Antibacterial effect of two fast-acting antipseudomonal antibiotics in combination

In view of the demonstrated inability of the antipseudomonal antibiotics in monotherapy to kill and prevent regrowth of resistant subpopulations, especially for the hypermutable strain, we examined a combination of two rapidly killing agents, tobramycin and ciprofloxacin. The time-course profiles of bacterial density as determined on antibiotic-free and antibiotic-containing plates for this combination are presented in **Fig. 3**. The time-course profiles for the

tobramycin and ciprofloxacin monotherapy arms of the combination study were in agreement with those from the monotherapy studies (**Fig. 1**).

Against PAO1, combinations with each of the three tobramycin concentrations and 1 mg/L ciprofloxacin exhibited synergy (>2 $\log_{10}$ CFU/mL killing compared to the most active monotherapy), whilst 4 mg/L ciprofloxacin in monotherapy was sufficient to suppress bacterial counts at 48 h. Notably, among all combinations, only the 1 and 4 mg/L tobramycin with 0.125 mg/L ciprofloxacin resulted in the emergence of less-susceptible populations to tobramycin (≤6.5 $\log_{10}$ CFU/mL), and ciprofloxacin (≤2.7 $\log_{10}$ CFU/mL). Against PAOΔ*mutS*, all tobramycin concentrations were synergistic in combination with 1 and 4 mg/L ciprofloxacin. The PAOΔ*mutS* exhibited less-susceptible populations to tobramycin for all treatments; however they were suppressed to values below that of the growth control for all combinations with 1 and 4 mg/L ciprofloxacin. No ciprofloxacin less-susceptible populations were observed for 4 and 8 mg/L tobramycin with 1 mg/L ciprofloxacin, and 8 mg/L tobramycin with 4 mg/L ciprofloxacin. Thus, the combination resulted in synergistic bacterial killing and the suppression of less-susceptible populations of PAOΔ*mutS*. These results are in agreement with a previous study in a murine model that found the tobramycin and ciprofloxacin combination was synergistic against PAOΔ*mutS* and resulted in reduced mortality and bacterial load without emergence of resistance [18]. The combination of tobramycin and ciprofloxacin appears promising against hypermutable *P. aeruginosa* due to the synergistic antibacterial activity and suppression of less-susceptible populations.

4. Conclusions

The current study systematically compared the antibacterial effects of monotherapy with a range of antibiotics having different mechanisms of action; we examined the time-course of bacterial counts and emergence of resistant populations of non-hypermutable and hypermutable *P. aeruginosa*. An additional component assessed the tobramycin and ciprofloxacin combination against these strains. This is the first study to have characterised the time-course of bacterial killing, regrowth and emergence of resistance of hypermutable PAOΔ*mutS* against multiple antibiotics over 48 h. We demonstrated that bacterial regrowth and emergence of resistant populations over 48 h were generally more pronounced for PAOΔ*mutS* than PAO1. In conclusion, our results indicate that monotherapy with clinically relevant concentrations of commonly used antipseudomonal antibiotics is not a viable option to combat hypermutable *P. aeruginosa* due to the resulting emergence of resistant populations. Tobramycin plus ciprofloxacin was identified as a promising combination for synergistic killing and suppression of resistant populations of a hypermutable *P. aeruginosa* strain. However, further studies in dynamic *in vitro* systems are warranted to rationally optimise this combination against infections caused by these difficult to treat hypermutable strains.

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Declaration of interest

Conflicts of interest: none.

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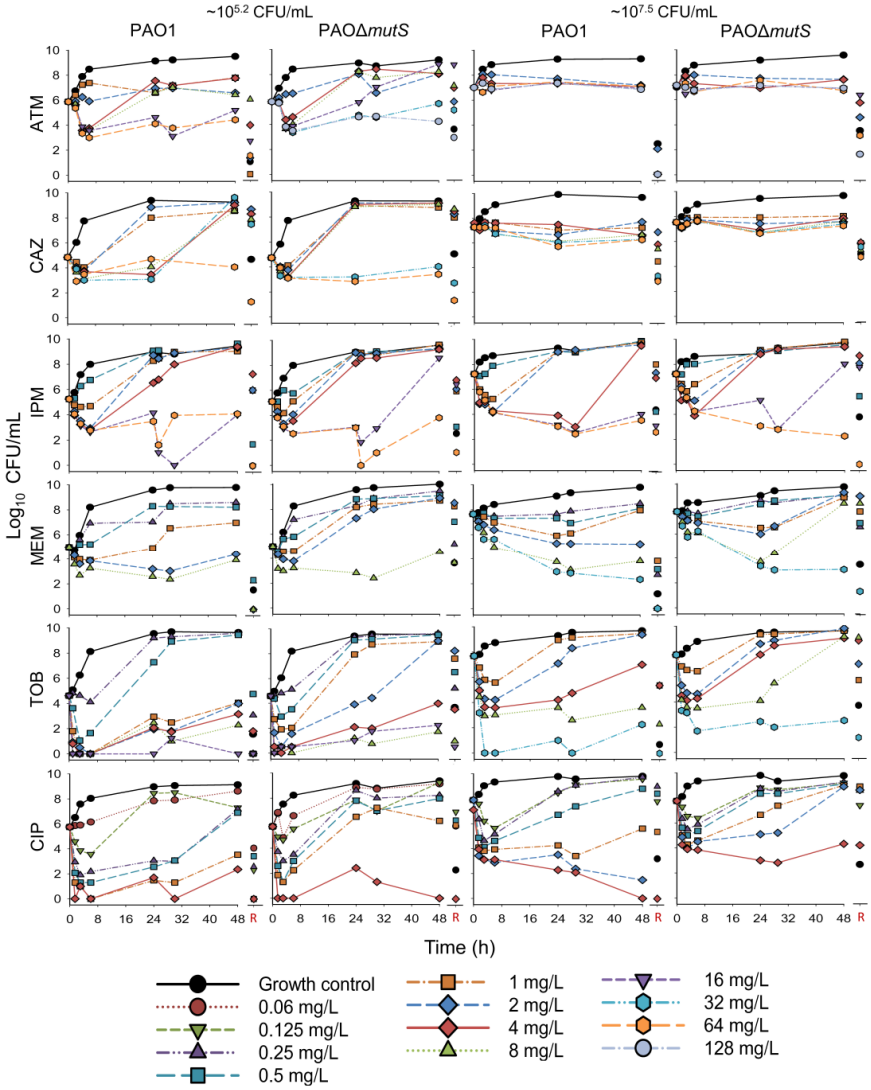

Fig. 1: The $\log_{10}$ viability counts (CFU/mL) of bacterial growth *vs*. time for non-hypermutable PAO1 and hypermutable PAOΔ*mutS* exposed to aztreonam (ATM), ceftazidime (CAZ), imipenem (IPM), meropenem (MEM), tobramycin (TOB) and ciprofloxacin (CIP) in 48-h static concentration time-kill experiments at two initial inocula (lower ~$10^{5.2}$ CFU/mL on the left and higher ~$10^{7.5}$ CFU/mL on the right). The following concentrations were studied against both inocula of each strain (unless denoted otherwise): aztreonam 1[a], 2, 4, 8[b], 16, 32[c], 64[a,d] and 128[c,d] mg/L; ceftazidime 1, 2, 4, 8, 32 and 64 mg/L; imipenem 0.5, 1, 2, 4, 16 and 64 mg/L; meropenem 0.25, 0.5, 1, 2, 8 and 32[d] mg/L; tobramycin 0.25[b], 0.5[b], 1, 2, 4, 8, 16[b] and 32[d] mg/L; and ciprofloxacin 0.0625[b], 0.125, 0.25, 0.5, 1, 2[d] and 4 mg/L.

R: denotes the bacterial population that grew on antibiotic-containing agar plates at 48 h.

[a] concentration was only used against the lower inoculum ($10^{5.2}$ CFU/mL) of PAO1.

[b] concentration was only used against the lower inoculum ($10^{5.2}$ CFU/mL) of both strains.

[c] concentration was only used against the lower inoculum ($10^{5.2}$ CFU/mL) of PAOΔ*mutS*.

[d] concentration was only used against the higher inoculum ($10^{7.5}$ CFU/mL) of both strains.

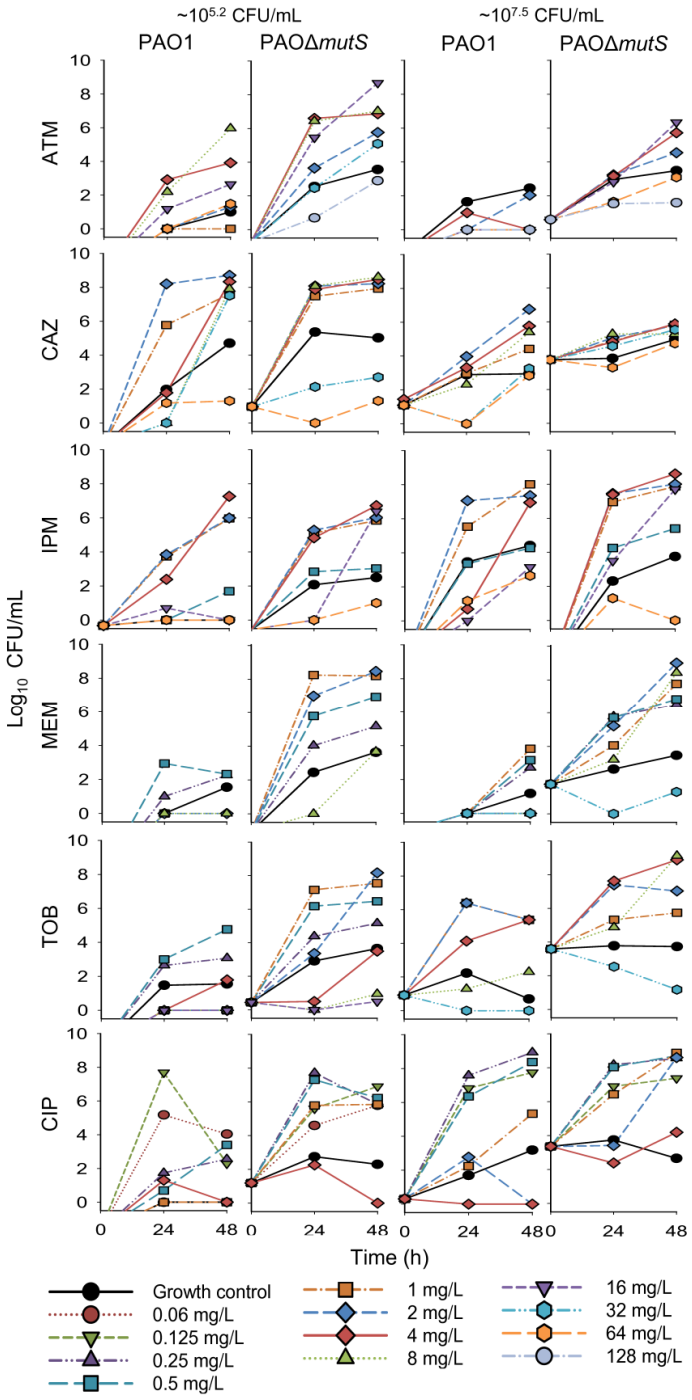

Fig. 2: The $\log_{10}$ viability counts (CFU/mL) of bacterial growth on antibiotic-containing agar plates (resistant populations) *vs*. time for non-hypermutable PAO1 and hypermutable PAOΔ*mutS* exposed to aztreonam (ATM), ceftazidime (CAZ), imipenem (IPM), meropenem (MEM), tobramycin (TOB) and ciprofloxacin (CIP) in 48-h static concentration time-kill experiments at two initial inocula (lower ~$10^{5.2}$ CFU/mL on the left and higher ~$10^{7.5}$ CFU/mL on the right).

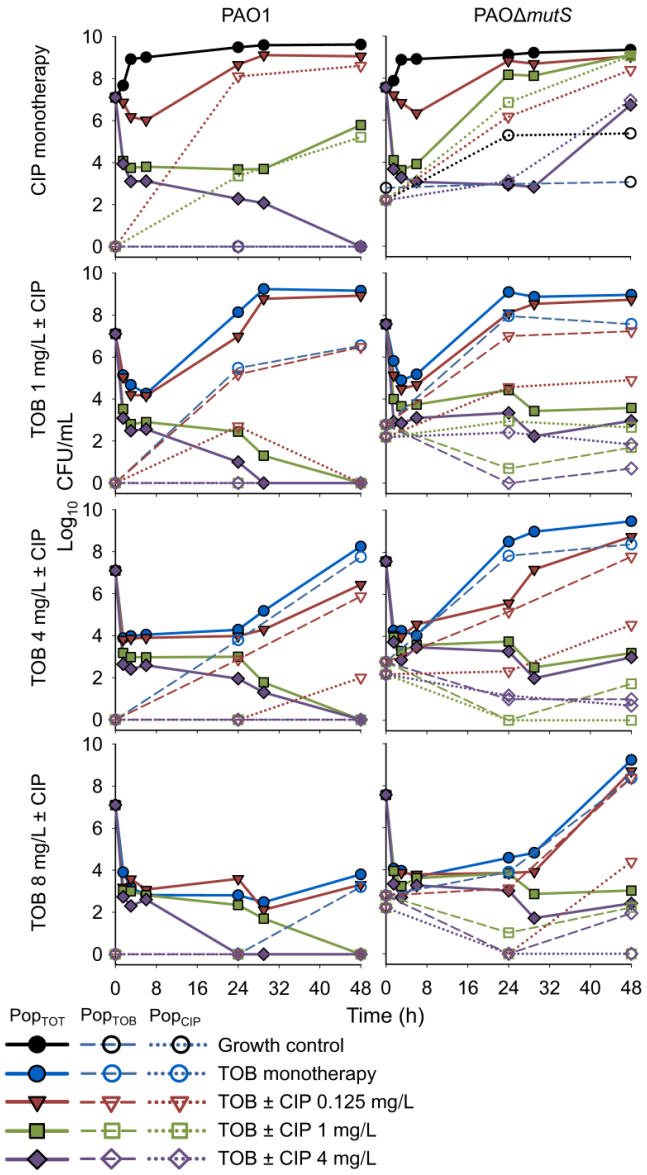

Fig. 3: The $\log_{10}$ viability counts (CFU/mL) of bacterial growth *vs*. time for non-hypermutable PAO1 (left) and hypermutable PAOΔ*mutS* (right) exposed to tobramycin (TOB) and ciprofloxacin (CIP) in mono- and combination therapy in 48-h static concentration time-kill experiments at an inoculum of ~$10^{7.5}$ CFU/mL. The top-tier panels show the growth control and ciprofloxacin monotherapies, the second-tier panels show the 1 mg/L tobramycin in monotherapy and combinations with ciprofloxacin, the third-tier panels show the 4 mg/L tobramycin in monotherapy and combinations with ciprofloxacin and the fourth-tier panels show the 8 mg/L tobramycin in monotherapy and combinations with ciprofloxacin. The solid lines and symbols represent the total population (Pop_{TOT}) and the hollow symbols with dashed and dotted lines are the tobramycin- (Pop_{TOB}; 2.5 mg/L) and ciprofloxacin-less susceptible (Pop_{CIP}; 0.625 mg/L) populations, respectively.

Chapter 5

Characterization of hypermutator *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis in Australia

Preamble

A logical step following on from **Aim 2** was to utilise clinical hypermutable strains to evaluate a promising antibiotic combination. However, to allow the best selection of the combination to be studied, susceptibilities to clinically important antibiotics required consideration. To address **Aim 3** we utilised susceptibility testing, followed by hypermutator determinations of a cohort of clinical *P. aeruginosa* isolates from the lungs of CF patients at the Alfred Hospital, Australia. We further characterised the hypermutable *P. aeruginosa* isolates using whole genome sequencing, phylogenetic analysis and bioinformatics to ascertain the causes of hypermutation. This Chapter supported **Hypothesis 3**; that hypermutable *P. aeruginosa* would be prevalent in respiratory infections of CF patients from an Australian clinic and show a high level of antibiotic resistance. Hypermutation in isolates from CF patients in Australia has not been examined for a decade. Previous studies have also only utilised a limited number of isolates from different, widespread geographical locations in Australia. This Chapter contains the first comprehensive characterisation of the largest collection of hypermutable *P. aeruginosa* isolates obtained more recently from CF patients with respiratory infections in an Australian clinic.

Manuscript

Characterisation of hypermutator *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis in Australia

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Running Head: Characterisation of Hypermutators in Cystic Fibrosis

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Abstract

Hypermutable *Pseudomonas aeruginosa* isolates (hypermutators) are often found in European cystic fibrosis (CF) patients with chronic respiratory infections and are associated with reduced lung function. Their prevalence has been well established amongst European CF patients, however has not been extensively investigated for Australian CF patients. Hypermutators display a greatly increased mutation rate and have an enhanced ability to become resistant to antibiotics during treatment. This study aimed to determine the prevalence of hypermutable *P. aeruginosa* isolates in patients with CF from Australia and characterise the genetic diversity and antibiotic susceptibility of these isolates. A total of 59 *P. aeruginosa* clinical isolates from patients with CF were characterised. For all isolates, rifampicin mutation frequencies (MF) and susceptibility to a range of antibiotics was determined. Of the 59 clinical isolates, 13 (22%) were hypermutable. Whole genome sequences were determined for all hypermutable isolates. Core genome polymorphisms were used to assess genetic relatedness of the isolates, both to each other and to a sample of previously characterised *P. aeruginosa* strains. Phylogenetic analyses showed that the hypermutators were from divergent lineages and the hypermutator phenotype was mostly the result of mutations in *mutL*, followed by *mutS*. Non-susceptibility to all tested antibiotics and multidrug resistance was greater for hypermutable than non-hypermutable isolates (MDR, 38% *vs*. 22%). Hypermutable isolates also contained a range of other mutations likely associated with adaptation of *P. aeruginosa* to the CF lung environment. Therefore, hypermutable *P. aeruginosa* play a critical role in the antibiotic resistance problem in CF patients from Australia.

Importance

Hypermutable *Pseudomonas aeruginosa* isolates are highly prevalent in chronic respiratory infections in European patients with cystic fibrosis. These isolates have a greatly increased mutation rate, are associated with decreased lung function in cystic fibrosis patients, and are linked with increased antibiotic resistance. There have been limited studies on the prevalence in patients with cystic fibrosis from Australia. This study identified the prevalence of hypermutable isolates within a cohort of cystic fibrosis patients from an Australian clinic and assessed the antibiotic resistance associated with their presence. Furthermore, the molecular mechanisms of hypermutator development and antibiotic resistance were examined.

Introduction

Pseudomonas aeruginosa frequently causes chronic pulmonary infections that are associated with increased morbidity and mortality among patients with reduced lung function, especially those with cystic fibrosis (CF) (1, 2). Patients with CF have frequently been found to harbour hypermutable *P. aeruginosa* isolates (3-6) and these isolates are generally associated with worsened patient outcomes (4). These hypermutators show an increased mutation rate of up to 1,000-fold compared to wild-type strains (6) and this, together with the short bacterial generation time, allows them to rapidly adapt to a variety of stressful environments (7). Hence, hypermutable *P. aeruginosa* can quickly adapt to antibiotic exposure (4, 7) and these strains have been strongly linked with antibiotic resistance within patients with CF (3, 4, 6, 8).

This hypermutator phenotype results from the mutation of genes involved in DNA repair, especially genes involved in the mismatch repair (MMR) system (*mutS*, *mutL* and *uvrD*) (9, 10). Mutations in the 7,8-dihydro-8-oxo-deoxyguanosine (8-oxodG or GO) system genes, *mutM*, *mutY* and *mutT*; oxidative damage prevention *oxyR* and superoxide dismutase *sodA* (*sodM* is the homologue in *P. aeruginosa*) genes; and *mutD*, have also been found to produce the hypermutator phenotype (3).

P. aeruginosa show a variety of diverse phenotypic changes associated with adaptation to the CF lung, including conversion to a mucoid phenotype (11, 12), inactivation of quorum sensing functions (13-15), motility loss (16, 17) and various auxotrophies (18). These phenotypic changes may occur more readily in hypermutator strains due to the increased mutation rates. There has been discordance in the literature over whether mucoid phenotype and quorum sensing are associated with hypermutator phenotype (19, 20). One of these studies found mutations in *mucA* and *lasR* occurred prior to the acquisition of the hypermutator phenotype of *P. aeruginosa*

isolates from CF patients (20). The mucoid phenotype occurs when there is increased alginate biosynthesis. This can result from inactivating mutations in the *mucA* gene, encoding the anti-sigma factor (σ^{22}), increased expression of the sigma factor (σ^{22}) *algU* (or *algT*) or various mutations within the multi-component signal transduction genes (*algR* or *algP*), which give rise to increased alginate production through the activation of the *algD* promoter (12).

The increased antibiotic resistance found with hypermutators from CF patients (3, 4, 6, 8) was proposed to be due to mutation driven adaptation (4, 7). Common mutation-mediated antibiotic resistance mechanisms include the increased expression of Mex-efflux pumps, following mutations in regulator genes (21-23), antibiotic binding-site modifications (24), increased production of antibiotic inactivating enzymes (25), and cell membrane permeability changes that lead to reduced intracellular drug accumulation through the inactivation of outer membrane porins (23).

The prevalence of hypermutators in isolates obtained from CF patients has been well examined throughout Europe (on average 27%); Denmark 37% (20, 26), United Kingdom 31% (19) , France 27% (8), Spain 21% (4, 27, 28), and Germany 18% (29). However, hypermutators in isolates obtained from CF patients in Australia have only been examined briefly. Previously, four isolates from CF patients in Australia were included in a European multicentre study; however only mutation frequencies were determined for these isolates and there was no description of their individual results (30). Another study assessed a wide spread clone, CC274, determining the phylogeny, interpatient dissemination, and hypermutator and resistance genotypes, which included eight isolates from different geographical locations in Australia obtained between 2007-2008 (31).

Given the importance of hypermutator *P. aeruginosa* strains for antibiotic resistance and worsened patient outcomes, we aimed to determine the prevalence of hypermutators out of isolates obtained more recently from chronically infected CF patients from Australia, and to characterise and evaluate the cause of hypermutation. We analysed 59 *P. aeruginosa* isolates from patients with CF and determined what percentage were hypermutators. Each of the isolates was tested for antibiotic susceptibility, and we used whole genome sequencing (WGS) with comparative genomic and phylogenetic analysis to further characterise the hypermutators. This is one of the first comprehensive characterisations of hypermutable *P. aeruginosa* isolates from CF patients in an Australian clinic that utilises WGS analyses of *P. aeruginosa* hypermutable isolates.

Results and discussion

Prevalence of* P. aeruginosa *hypermutators in CF patients from Australia and resistance profiles. Out of a total of 59 *P. aeruginosa* isolates (designated CW1 – CW59) from 37 patients with CF who attended the Alfred Hospital in 2013, 13 (22%) were found to be hypermutable and 10 patients (27%) were found to harbour them (Table 1). These hypermutators included three paired isolates (CW30 and CW31, CW34 and CW35, CW41 and CW42), where each isolate of the pair was recovered from the same patient (on the same date). Our results were comparable with a multicentre study in North America that reported 17% of isolates and 31% of patients were harbouring these hypermutators (32). Our results were also very similar to studies in Spain, with on average 21% (with a range of 15% – 29%) of isolates and 30% (with a range of 24% – 37 %) of patients harbouring hypermutators (4, 27, 28). All of the isolates defined as hypermutable showed at least a 48-fold increase in MF compared to PAO1, while the highest MF in the non-hypermutator isolates was 8-fold that of PAO1. Furthermore, analysis of total number

of mutants per 10^8 bacterial cells (**Fig. 1**) showed that the described 20-fold definition of hypermutation (4) allows a very clear distinction between hypermutable and non-hypermutable isolates. Of the 13 hypermutable isolates, nine showed more than a 100-fold MF increase, with CW35 showing the highest MF of 470-fold that of PAO1.

The Etest MIC values and MDR based on EUCAST susceptibility breakpoints (33), with MDR described as non-susceptible to ≥1 agent from ≥3 different antimicrobial categories (34), is displayed in **Table 1**. MDR strains were more abundant among hypermutators (38%) than non-hypermutators (22%) in agreement with a previous study (8), although this difference did not reach statistical significance (Fisher's exact test, $P = 0.192$). Among hypermutable isolates, the percentage of non-susceptible isolates determined using Etest was 77% for ciprofloxacin, 46% for ceftazidime, aztreonam and meropenem, and 38% for tobramycin. In non-hypermutable isolates, the percentage of non-susceptible isolates was lower for each antibiotic; ciprofloxacin, 65%; aztreonam, 33%; tobramycin, 22%; ceftazidime, 17%; and meropenem, 17%. These results are in agreement with previous studies that found greater resistance to antimicrobial agents for hypermutators compared to non-hypermutators (4, 35).

Phylogenomic analysis. The evolutionary relatedness of the 13 hypermutable clinical isolates, determined using draft WGS of all 13 isolates and compared these with 62 publicly available *P. aeruginosa* genomes (including PAO1 and ATCC 27853 wild-type strains) is displayed in **Fig. S1**. A maximum likelihood phylogeny was determined by comparison of all core-genome single nucleotide polymorphisms (cgSNPs). The 13 hypermutable isolates were spread widely across the phylogenetic tree, indicating that they arose from diverse *P. aeruginosa* strain backgrounds. Of the three paired isolates, each of the individual isolates of a pair clustered closely together, indicating that they are likely to be true isogenic derivatives. Indeed, the

average number of cgSNPs between all of the non-paired hypermutator isolates was 24,640 while the average number of cgSNPs between the paired isolates was 774. The three different pairs were not closely related, indicating that they each arose from different backgrounds. CW8 and CW13 were found to be clustered near an Australian Epidemic Strain, AES-1R (36). CW41 and CW42 were near Denmark Epidemic Strain DK2 (37, 38) and CW5 and CW45 were near the wild-type PAO1 (**Fig. 2**). Similarities with *P. aeruginosa* genomes from different environments around the world demonstrated diversity between these isolates as shown in **Fig. S1**.

Mutations responsible for the hypermutator phenotype. Genes likely to be involved in the hypermutator phenotypes, based on analysis of the draft WGS of the 13 hypermutable isolates, are presented in **Fig. 2**. For seven of the 13 hypermutable isolates we could identify clearly inactivating mutations, notably all of them in *mutL* and *mutS*, that are likely responsible for the increased mutation rate (**Table 2**). Six of the isolates had inactivating *mutL* mutations; CW8, CW19 and CW45 had frameshift mutations, CW30 and CW31 had an identical nonsense mutation and CW44 had a large deletion. Isolate CW5 had a frameshift mutation within *mutS*. For the other six isolates there were no clearly inactivating mutations identified, but we predict that the hypermutator phenotype is due to single amino acid missense mutations in highly conserved regions of critical repair proteins (**Table 2**) for the following reasons. Isolate CW13 had single base changes resulting in missense mutations in both MutL and UvrD, CW34 and CW35 had missense mutations within MutS, and CW41 and CW42 had missense mutations within MutL. The A470V mutation observed in the CW13 MutL has not been found in a collection of other *P. aeruginosa* MutL sequences although it has been observed in the *Escherichia coli* MutL (39). The K83E mutation in the CW41 and CW42 MutL sequences lies

within the ATPase domain (ATPlid) of MutL (40). Therefore, we predict that the MutL proteins of CW13, CW41 and CW42 are likely non-functional or show highly reduced activity. The CW34 and CW35 missense mutations (C224R & T287P) in MutS were observed in two other hypermutator *P. aeruginosa* strains obtained from CF patients in Australian and German clinics; these studies demonstrated that the MutS mutations were directly responsible for the hypermutator phenotype as complementation with intact MutS restored the non-hypermutator function (29, 31). Interestingly, CW30, CW31, CW41, CW42 and CW44 all had identical missense mutations (S662N and N666S, data not shown) in UvrD that another study has found previously in paired isolates, where one was a hypermutator and the other a non-hypermutator (29). Furthermore, the CW28 single mutation resulting in an E236D substitution (data not shown) in MutT was also observed in CW34, CW35, CW41, CW42 and CW44; however, this residue is not highly conserved (31, 41). Hence we assume these mutations are unlikely to produce the hypermutator phenotype. Two isolates (CW5 and CW45) had mutations leading to missense substitution mutations in SodM (G109E), although both of these isolates also had clearly inactivating mutations in other critical mismatch repair genes (*mutS* and *mutL*, respectively). Isolate CW34 had a missense mutation in OxyR (L148P), the primary hydrogen peroxide sensing transcriptional activator responsible for hydrogen peroxide resistance (42), and isolate CW8 had a missense mutation in PolA (E825K), both of which were in conserved amino acids, suggesting that these proteins are likely non-functional or exhibit reduced function.

The observation that mutations in *mutL* were the most frequent cause of hypermutability is in agreement with another study where 60% of hypermutable isolates were attributed to mutations in *mutL*, and the remaining 40% to *mutS* (35). However, other studies have found that hypermutation was more frequently due to a defective *mutS* (6, 43). Previous studies on

hypermutator isolates from CF patients have identified mutations in the MMR system genes, *mutS*, *mutL* or sometimes *uvrD* resulting in the hypermutator phenotype (29, 32, 43). In contrast, another study identified mutations in *mutT* and *mutY* in hypermutable *P. aeruginosa* isolates from CF patients (44). In the present study there were no inactivating mutations observed in the GO system genes *mutT*, *mutY* and *mutM*. However, mutations present in *mutT* (L236D) and *mutM* (D61N and L132P), along with *polA* (D876E; data not shown), have been previously observed in both hypermutator and non-hypermutator isolates (31). Therefore, we assume these mutations are unlikely to result in hypermutator phenotype. As expected, we identified no mutations in *mutD*, encoding the epsilon subunit of DNA polymerase III (3, 45); no naturally occurring hypermutator strains with *mutD* mutations have ever been identified, likely due to the highly reduced growth rate of these strains (3).

Mutations responsible for the mucoid phenotype and antibiotic resistance. The overview of gene mutations involved in mucoid phenotype and antibiotic resistance are shown in **Fig. 2** and the mutations involved in non-functional genes are in **Table S1** (online supplement). Mucoid phenotype is a common trait of *P. aeruginosa* obtained from CF lungs (20, 35, 46). We observed growth of our collection of 59 isolates and recorded whether mucoid phenotype was exhibited. We found that 25 isolates (42%) exhibited a stable mucoid phenotype with 24 of the 25 being non-hypermutators (Table 1). The stable mucoid, hypermutable CW19 exhibited clearly inactivating mutations in two mucoid phenotype associated genes; a nonsense mutation in *mucA* (encoding the anti-sigma factor) and a large amino acid deletion in the alginate transcriptional regulator, *algP*. CW5, CW13, CW31, CW35, CW41 and CW44 all showed an unstable conversion to mucoid phenotype. Non-stable mucoid and nonmucoid hypermutator isolates also contained inactivating mutations in a range of mucoid phenotype related genes. Over half (54%)

of the hypermutable isolates had clearly inactivating mutations in *mucA*; CW5, CW41, CW44 and CW45 (nonmucoid) had frameshift mutations (CW41 and CW44 had an identical frameshift mutation) and the paired isolates CW30 (nonmucoid) and CW31 had an identical nonsense mutation. CW5, CW13 and CW45 had clearly inactivating frameshift mutations in *algP*. CW13 also exhibited a missense substitution in the sigma factor AlgU that likely stopped the conversion to mucoid from being stable. None of the hypermutable isolates had changes in the alginate biosynthetic gene, *algD*. CW35 had no inactivating mutations in the genes investigated suggesting mutations in other alginate biosynthesis or regulatory genes may have resulted in this unstable mucoid conversion. The unstable mucoid phenotype observed in some of these isolates is not uncommon for a defective *mucA*, as mutations in this gene are not always stable (47). Only CW30 and CW45 showed a defective *mucA* with no observable mucoid conversion displayed.

Four hypermutable isolates (CW30, CW31, CW41 and CW42) had no identifiable *lasR* (**Fig. 2**), which encodes the major quorum sensing regulator (14). Three hypermutable isolates had clearly inactivating mutations in *lasR*; CW28 had a frameshift mutation, whilst CW5 and CW45 had an identical nonsense mutation. CW19, CW34, CW35 and CW44 all had missense mutations in the DNA binding domain of LasR with the paired isolates CW34 and CW35 displaying an identical missense mutation. These mutations likely result in non-functional or highly reduced quorum sensing activity (48). A limitation of this study is that we cannot determine the order of appearance of the mutations in our isolates to comment on whether the acquisition of the hypermutator phenotype or the mutations came first.

We analysed the WGS of the hypermutable stains for mutations associated with specific antibiotic resistance mechanisms (**Fig. 2** & **Table S1**). Firstly, we examined the repressors of the four main efflux systems of *P. aeruginosa; mexR, armR* (antirepressor for *mexR*)*, nfxB, mexZ*

and mexT. Clearly inactivating mutations were found in *mexR*, *mexT*, *mexZ* and *nfxB*, which we predict would result in increased expression of MexAB-OprM, MexEF-OprN, MexXY-OprM and MexCD-OprJ efflux pumps, respectively (49). CW19 had a clearly inactivating frameshift mutation in *nfxB*, and CW28 and CW44 had frameshift mutations in *mexT*. Seven hypermutable isolates had clearly inactivating mutations in *mexZ*; CW5, CW28, CW34, CW35 and CW45 had frameshift mutations (CW5 and CW45, and the paired isolates CW34 and CW35 had identical frameshift mutations) whilst CW41 and CW42 had an identical nonsense mutation. Both CW13 and CW19 had mutations in the *mexZ* gene resulting in the addition of two amino acids in a conserved region and a missense mutation, respectively. Both changes likely gave rise to non-functional or reduced function proteins and the subsequent increased expression of the MexXY-OprM efflux pump. High-level aminoglycoside resistance can result due to the overexpression of the MexXY-OprM multi-drug efflux pump (11, 22). Our study showed that all isolates with increased tobramycin MIC values (based on EUCAST susceptibility breakpoints (50)) also contained clearly inactivating mutations within the MexXY-OprM repressor, *mexZ*. This is similar to a previous study that found that 82% of multidrug-resistant *P. aeruginosa* isolates had an overproduction of MexXY-OprM (51). Missense mutations were found from single base changes in *mexT*, *nfxB* and *armR* (the antirepressor for *mexR*) that likely resulted in proteins that are non-functional or exhibit reduced function, causing the subsequent up-regulation of the respective efflux pumps. All hypermutable isolates showed no changes in *mexR*, and therefore should have normal expression of the MexAB-OprM efflux pump. However, expression levels of efflux pumps cannot be ascertained from this data. Ultimately, transcriptomic studies are required to elucidate the expression levels and full effect of these mutations.

We also examined the gene *oprD*, which encodes the common outer membrane porin OprD, whose altered expression is responsible for reduced susceptibility to carbapenems (52). All of the isolates were found to have clearly inactivating mutations in *oprD* regardless of meropenem resistance (**Fig. 2** and **Table 1**). Meropenem-resistant CW5 and CW45 had an identical nonsense mutation in *oprD*. All other isolates had deletions in *oprD* resulting in frameshifts, except for the meropenem-resistant CW13 that had no resulting frameshift. The frameshift in CW8 reverted to the correct frame through other deletions. The meropenem-susceptible paired isolates CW30 and CW31 had an identical amino acid loss, as did the paired CW41 and CW42 isolates, although only CW42 was meropenem resistant. The clearly inactivating mutations in *oprD* observed alone for a number of hypermutators did not always lead to greatly reduced susceptibility. This is in agreement with reports that a greatly reduced meropenem susceptibility requires multiple resistance mechanisms, including AmpC overproduction and overexpression of MexCD-OprJ, MexXYOprM and especially MexAB-OprM efflux pumps, to be up-regulated (51). Meropenem-resistant CW5, CW28 and CW45 also had large additions or losses of nucleotides in *nfxB* that likely contributed to the observably reduced susceptibility. However transcriptomic analysis of these isolates would be required to confirm this.

Fluoroquinolone resistance is usually associated with mutations in the genes *gyrA*, *gyrB*, *parC* and *parE*, encoding fluoroquinolone targets (53, 54). We identified mutations within these genes in many isolates (**Fig. 2**). All isolates (except for CW19, CW31 and CW44) were found to be ciprofloxacin-resistant (**Table 1**); this was likely due to previously described missense mutations in GyrA residues 83 and 87 for all isolates (55, 56), except CW28 which showed a missense mutation in GyrB (E468D) that likely caused the increase in ciprofloxacin MIC.

However, a previously described missense mutation in GyrB (S466F) (31) that was found in CW31, was not matched with an increase in ciprofloxacin MIC. Furthermore, the missense mutations found in GyrA of CW19 and GyrB of CW44, which were found to be in conserved regions, were likely not in important regions due to the ciprofloxacin MIC not being elevated.

In conclusion, in this work we have identified the prevalence of hypermutators and established that the hypermutator *P. aeruginosa* isolates come from divergent lineages, with true isogenic derivatives amongst them. Furthermore, mutations in *mutL* and *mutS* were determined to be the cause of the hypermutator phenotype in the majority of these isolates. Hypermutable isolates had a higher proportion of MDR and were more often resistant to each of the tested antibiotics in comparison to non-hypermutable isolates. Therefore, hypermutable *P. aeruginosa* are playing a critical role in the antibiotic resistance problem in CF patients from Australia.

Materials and methods

Bacterial strains and media. We characterised 59 *P. aeruginosa* clinical isolates collected from 37 cystic fibrosis patients (in 2013) (**Table 1**). The *P. aeruginosa* PAO1 WT strain and the hypermutable PAOΔ*mutS* strain (*mutS* knock out of PAO1) (32) were used as controls with these clinical isolates. All susceptibility studies and viable counting were performed on cation-adjusted Mueller Hinton agar (CAMHA; containing 25 mg/L Ca^{2+} and 12.5 mg/L Mg^{2+}; Medium Preparation Unit, The University of Melbourne, Parkville, Australia). Drug-containing agar plates were prepared using CAMHA (BD, Sparks, MD, USA) supplemented with the appropriate amount of antibiotic. The rifampicin (Sigma-Aldrich, Sydney, Australia) stock solution was prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Sydney, Australia) and subsequently filter-sterilised using a 0.22 µm PVDF syringe filter (Merck

Millipore, Cork, Ireland). The mucoid or non-mucoid phenotype of growth exhibited after 24 h incubation at 37ºC on antibiotic-free CAMHA of the 59 clinical isolates was recorded.

Mutation frequency determination. Rifampicin mutation frequencies (MF) were determined as previously described (4). This was carried out at a minimum in triplicate and the average was used as the mutation frequency.

Antibiotic susceptibility. The MICs of ceftazidime (0.016 – 256 mg/L), ciprofloxacin (0.002 – 32 mg/L), meropenem (0.002 – 32 mg/L), aztreonam (0.016 – 256 mg/L) and tobramycin (0.016 – 256 mg/L) for each clinical isolate were determined using Etest strips (bioMérieux, North Ryde, Australia). The manufacturer's protocol was followed with alterations as described previously (28) and readings taken at 20, 24, 36 and 48 h for comparison. The 24 h reading was used as the MIC, for slow growing isolates the first 3 time points were compared to ensure the most accurate representation of the MIC was chosen. The MICs were evaluated based on EUCAST susceptibility breakpoints (50) to elucidate which isolates were multidrug-resistant (MDR; previously described definition of MDR: resistant to ≥1 agent from ≥3 different antimicrobial categories (34)).

Whole genome sequencing. Genomic DNA (gDNA) was purified from each hypermutable clinical isolate using the GenElute™ Bacterial Genomic DNA kit (NA2110-IKT; Sigma-Aldrich, Castle Hill, NSW, Australia) using the manufacturer's protocol, except that 40 µL of sterile distilled H_2O was used in replacement of the elution solution. The gDNA was sequenced using the paired-end 150-bp protocol on an Illumina NextSeq at Micromon Next-Gen Sequencing Facility (Micromon, Monash University, Clayton, VIC, Australia).

De novo assembly of the raw read data (average read depth of 59371, range 501 – 600345) was performed using CLC Genomics workbench V7.03 software. Automatic

annotations of the *de novo* assemblies were produced using Rapid Annotation using Subsystem Technology (RAST), version 2, accessed November 2016 (57).

Phylogenetic analysis. The comparative phylogenetic analysis was performed on the *de novo* assembled genomes against 62 publicly available *P. aeruginosa* genomes obtained from Pathosystems Resource Integration Centre (PATRIC) (58). The phylogenetic tree to determine evolutionary relatedness was created using Parsnp, a fast core-genome multi-aligner software (Harvest) (59). Furthermore a SNP analysis were performed *via* Nullarbor V1.01 (60).

Comparative genomic analysis. The unmapped, paired reads were referenced assembled against PAO1 using the CLC Genomics Workbench. The nucleic acid sequences were extracted for the MMR genes, *mutS*, *mutL*, *mutT*, *mutY*, *mutM*, *mutD*, *uvrD* (*mutU*), *radA*, *pfpI*, *sodM*, *oxyR*, and *polA*; the mucoid phenotype genes, *mucA*, *algP*, *algU* and *algD*; the quorum sensing gene, *lasR*; and resistance genes, *gyrA*, *gyrB*, *parC*, *parE*, *armR*, *mexR*, *mexT*, *mexZ*, *nfxB*, and *oprD*. These nucleic acid sequences were converted to amino acid sequences using the ExPasy online translational tool on the Bioinformatics Research Portal (Swiss Institute of Bioinformatics); subsequently the amino acid sequences from each isolate were compared to PAO1 using the Clustal Omega multiple sequence alignment program (61-63). Frameshifts, mutations within conserved regions and the absence of amino acids or whole genes were located and analysed for proposed loss of function.

Statistics

Fisher's exact test and the Mann-Whitney U test were used for the comparison of resistance rates and distributions of MICs, respectively, between hypermutators and non-hypermutators. A P value of < 0.05 was considered statistically significant.

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Table 1: The bacterial characteristics of 59 clinical *P. aeruginosa* isolates from cystic fibrosis patients.

Isolate[a]	Mucoid[b]	Isolate pair	RIF MF (fold-change)[c]	ATM MIC [d,e]	CAZ MIC [d,f]	CIP MIC [d,g]	MEM MIC [d,h]	TOB MIC [d,e]
CW1	N		0.8	4	>256	0.75	8	12
CW2	N		0.4	0.016	0.032	0.047	0.016	2
CW3	M		6.2	0.125	0.125	0.25	0.25	0.75
CW4	N		1.5	8	2	1.5	0.25	6
CW5	N[i]		285.2	6	>256	1.5	>32	3
CW6	M		0.5	24	>256	4	12	3
CW7	N		1.2	1.5	2	2	2	0.032
CW8	N		141.0	1.5	0.75	4	1.5	0.75
CW9	N		1.0	16	4	0.25	0.19	0.75
CW10	N		1.0	6	>256	4	0.125	0.75
CW11	M		2.3	8	12	0.094	0.75	3
CW12	M		2.2	0.19	0.5	0.75	0.125	1
CW13	N[i]		180.5	0.5	24	4	8	1.5
CW14	M		0.6	0.047	0.25	1.5	0.125	1
CW15	M		4.4	4	1.5	2	0.094	1.5
CW16	M		4.8	0.125	0.75	0.75	0.125	3
CW17	N		7.5	0.5	4	4	4	3
CW18	M		3.2	0.19	1	1.5	0.064	1
CW19	M		76.9	1	16	0.5	0.75	1
CW20	N		0.7	8	>256	0.25	16	8
CW21	M		2.7	0.125	0.5	2	0.5	1.5
CW22	N		0.5	12	16	1	>32	32
CW23	M		2.6	0.064	0.25	0.5	0.032	0.38
CW24	N		1.0	0.5	1	0.5	0.047	2
CW25	M		1.9	0.032	0.125	0.75	1.5	1
CW26	N		1.4	0.75	2	1.5	>32	24
CW27	M		1.9	0.19	0.38	2	2	3
CW28	N		125.9	12	>256	3	>32	3
CW29	M		1.0	0.75	2	0.19	4	2
CW30	N	CW31	84.9	0.032	0.064	1	0.016	0.75
CW31	N[i]	CW30	143.9	0.064	0.094	0.094	0.004	1
CW32	N		2.8	16	12	1.5	>32	2
CW33	M		6.2	0.19	0.75	0.75	0.125	3
CW34	N	CW35	240.0	0.38	1	4	0.5	8
CW35	N[i]	CW34	472.5	0.19	1.5	3	0.5	8
CW36	N		6.5	0.75	4	1.5	0.75	>256
CW37	M		2.3	0.5	0.38	0.5	0.125	>256

CW38	M		1.7	0.75	0.75	0.5	0.25	3
CW39	M		1.1	4	1	2	0.5	16
CW40	M		2.1	1	0.75	>32	0.64	24
CW41	N[i]	CW42	48.4	0.5	0.75	4	0.75	16
CW42	N	CW41	54.5	2	>256	6	>32	48
CW43	M		0.9	0.19	0.5	0.25	1	3
CW44	N[i]		122.6	1.5	2	0.19	0.5	1
CW45	N		232.5	16	>256	1.5	>32	8
CW46	M		0.8	3	8	1.5	2	2.5
CW47	N		5.4	0.094	0.125	0.25	0.023	4
CW48	M		4.4	0.023	0.125	0.5	0.23	>256
CW49	N		3.1	0.19	0.75	0.094	0.32	4
CW50	M		3.0	0.19	0.75	0.125	0.125	3
CW51	N		4.2	0.19	0.5	0.5	0.032	2
CW52	N		2.7	0.25	0.5	0.75	0.032	1.5
CW53	M		7.8	0.38	2	4	0.19	1.5
CW54	N		0.4	0.5	2	8	3	1.5
CW55	N		0.7	32	>256	6	0.125	1.5
CW56	M		5.1	0.38	0.5	4	0.125	3
CW57	N		1.7	12	0.75	4	0.38	1
CW58	N		2.0	0.125	0.75	1	1.5	2
CW59	N		1.8	0.125	0.75	1	1.5	2

[a] Hypermutator strains are shown in purple
[b] Mucoid phenotype; M, mucoid; N, non-mucoid
[c] Mutation frequencies (MF) were determined as the fraction of the resistant population quantified on rifampicin (300 mg/L) containing CAMHA plates compared to the total population quantified on drug free CAMHA plates. Hypermutation was defined by the rifampicin MF being ≥ 20-fold higher than the PAO1 rifampicin MF.
[d] MICs indicative of resistance are shown in red and MICs indicative of intermediate resistance are shown in yellow. Antibiotics tested were; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; MEM, meropenem; TOB, tobramycin
[e] Hypermutators were not significantly more resistant, Mann-Whitney U exact test, $P > 0.05$
[f] Hypermutators were significantly more ceftazidime resistant; Mann-Whitney U exact test, $P = 0.019$
[g] Hypermutators were almost significantly more ciprofloxacin resistant, Mann-Whitney U exact test, $P = 0.062$
[h] Hypermutators were significantly more meropenem resistant, Mann-Whitney U exact test, $P = 0.05$
[i] Found to exhibit mucoid phenotype at times

Table 2: The mutations predicted to be involved in the hypermutator phenotype of each of the 13 hypermutable isolates.

Isolate [a]	Gene	Mutation	Predicted protein change
CW5	***mutS***	**13 bp deletion of nucleotides 1312-1324**	**Frameshift**
	sodM	GGG→GAG (G109E)	One amino acid substitution at a highly conserved amino acid
CW8	***mutL***	**4 bp deletion of nucleotides 700-703**	**Frameshift**
	polA	GAG→AAG (E825K)	One amino acid substitution at a highly conserved amino acid
CW13	*mutL*	GCG→GTG (A470V)	One amino acid substitution at a highly conserved amino acid
	uvrD	TGG→CGG (W146R)	One amino acid substitution at a highly conserved amino acid
CW19	***mutL***	**1 bp insertion (C) after nucleotide 1444**	**Frameshift**
CW30	***mutL***	**Codon 341 CAG→TAG**	**Mutation Q341STOP codon**
CW31	***mutL***	**Codon 341 CAG→TAG**	**Mutation Q341STOP codon**
CW34	*mutS*	TGC→CGC (C224R) ACC→CCC (T287P)	Two amino acid substitution at a highly conserved amino acids
	oxyR	CTG→CCG (L148P)	One amino acid substitution at a highly conserved amino acid
CW35	*mutS*	TGC→CGC (C224R) ACC→CCC (T287P)	Two amino acid substitution at a highly conserved amino acids
CW41	*mutL*	AAG→GAG (K83E)	One amino acid substitution at a highly conserved amino acid
CW42	*mutL*	AAG→GAG (K83E)	One amino acid substitution at a highly conserved amino acid
CW44	***mutL***	**378 nucleotide deletion after nucleotide 690**	**126-amino-acid deletion after amino acid 230**
CW45	***mutL***	**1 bp deletion at nucleotide 565**	**Frameshift**
	sodM	GGG →GAG (G109E)	One amino acid substitution at a highly conserved amino acid

[a] Clearly inactivating mutations are shown in bold

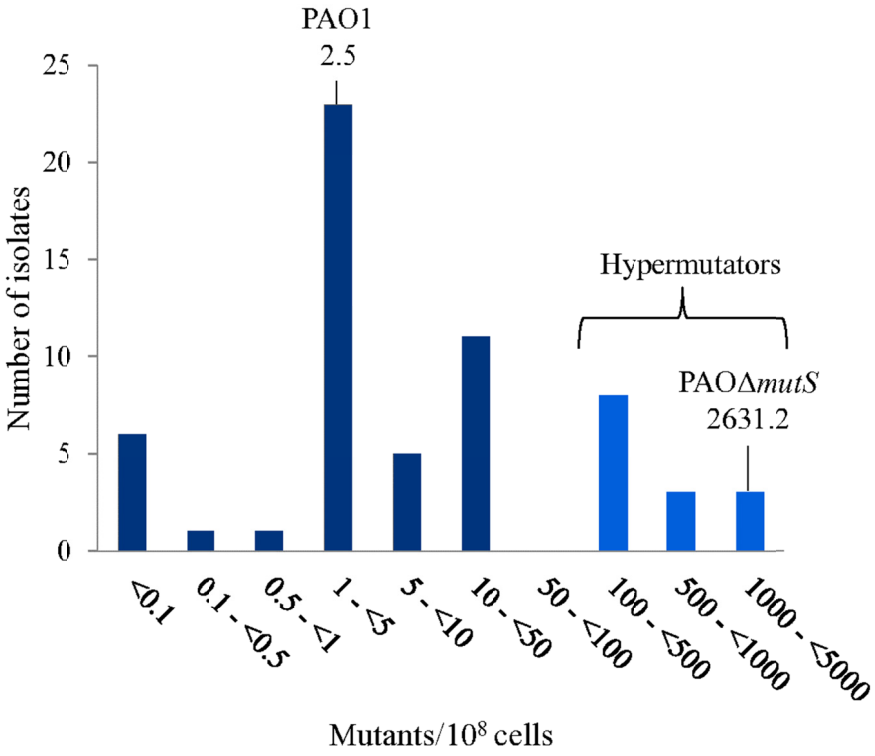

Fig. 1: The number of isolates for each range of rifampicin resistant mutants for every 10^8 bacterial cells determined for each of the 59 clinical isolates and PAO1 and PAOΔ*mutS*.

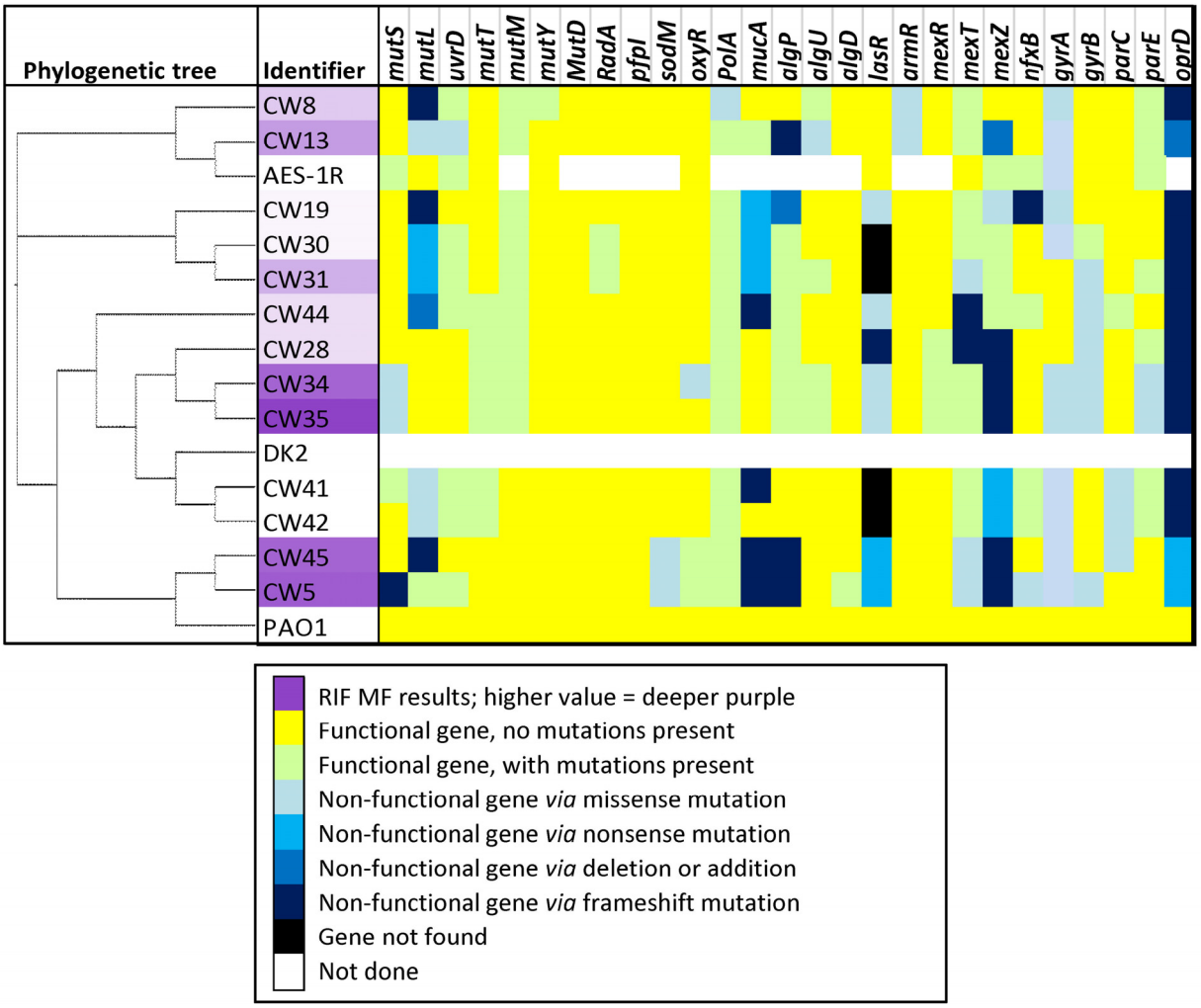

Fig. 2: The 13 hypermutable *P. aeruginosa* clinical isolates from cystic fibrosis patients, 2 previously characterised CF isolates (DK2 and AES-1R) and the well characterised PAO1 wild-type strain in a phylogenetic tree, revealing the strongest hypermutators with the deepest purple for high rifampicin mutation frequency (RIF MF), the descriptive functionality of each gene examined through whole genome sequencing and bioinformatics analysis.

Supplementary material

Table S1: The mutations predicted to be involved in reduced function or non-functional mucoid phenotype, quorum sensing and antibiotic resistance.

Strain	*mucA*	*algP*	*algU*	*lasR*	*armR*	*mexT*	*mexZ*	*nfxB*	*gyrA*	*gyrB*	*parC*	*parE*	*oprD*
CW5	-2bp nt421, 447; FS	-4bp nt835-838; FS		GAG→TA G; E145STOP		D253N; Δ1aa in CR	+2bp after nt343; FS	F96S; Δ1aa in CR	T83I; Δ1aa in CR	R137C; Δ1aa in CR			GAG→TA G; E176STOP
CW8					Y32C; Δ1aa in CR				D87N; Δ1aa in CR				Large deletions; FS, no FS
CW13		-13bp nt540-552; FS	R 66 C; Δ1aa in CR		Y32C; Δ1aa in CR		+6bp after nt160; +2aa in CR		T83I; Δ1aa in CR				Large deletions; no FS
CW19	349CAA→ TAA; Q117STO P	Large deletion		N216H; Δ1aa in CR			V23G; Δ1aa in CR	-5bp nt51-54, 57; FS	Y267H, P554S; Δ2aa in CR				-1bp after nt265; FS
CW28				-5bp (nt716-720; FS		-1bp nt234; FS	-13bp after nt334; FS			E468D; Δ1aa in CR			Large deletions, FS
CW30	CAG→TA G 367; Q123STO P			Absent					T83A; Δ1aa in CR				Large deletions, FS
CW31	CAG→TA G 367; Q123STO P			Absent		F272L; Δ1aa in CR				S466F; Δ1aa in CR			Large deletions, FS
CW34				T180I; Δ1aa in CR			-1bp nt530; FS		T83A, T325I;	Q467R; Δ1aa in CR		P438S; Δ1aa in CR	Large deletions,

						Δ2aa in CR				FS
CW35			T180I; Δ1aa in CR		-1bp nt530; FS	T83A, T325I; Δ1aa in CR	Q467R; Δ1aa in CR		P438S; Δ1aa in CR	-2bp after nt880; FS
CW41	-1bp nt428; FS		Absent		CAA→TAA; Q164STOP	T83I; Δ1aa in CR		R243C; Δ1aa in CR		+1bp after nt102; FS
CW42			Absent		CAA→TAA; Q164STOP	T83I; Δ1aa in CR		R243C; Δ1aa in CR		+1bp after nt102; FS
CW44	-1bp nt428; FS		K220N; Δ1aa in CR	-4bp nt231-234; FS			Met463I; Δ1aa in CR			Large deletions, FS
CW45	-1bp nt447; FS	+1bp after nt 822, -15bp nt825-839; FS,	GAG→TAG; E145STOP	D253N; Δ1aa in CR	+2bp after nt343; FS	T83I; Δ1aa in CR		D108G; Δ1aa in CR		GAG→TAG; E176STOP

nt, nucleotide; FS, frameshift; no FS, another deletion or addition returns the gene to frame; -, deletion; +, addition; Δ, substitution/change in nucleotide or amino acid; CR, conserved region

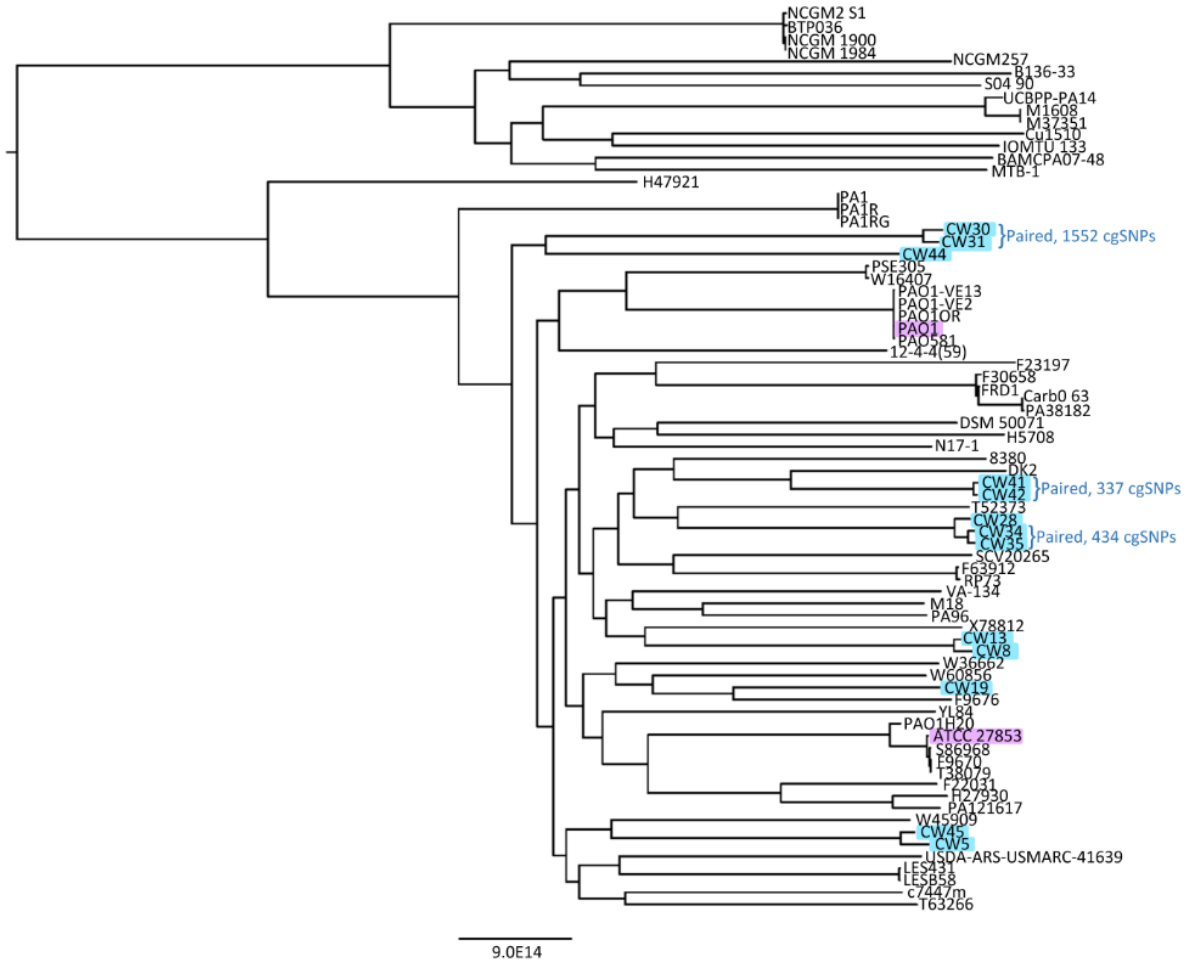

Fig. S1: The phylogenetic tree of the 13 hypermutable clinical isolates (highlighted in blue), reference strains PAO1 and ATCC 27853 (highlighted in purple), and 70 (including isolates from respiratory infections of CF patients) *P. aeruginosa* strains. Paired isolates are marked and their core genome single nucleotide polymorphisms (cgSNPs) are described.

Chapter 6

Meropenem in combination with ciprofloxacin combats hypermutable *Pseudomonas aeruginosa* from respiratory infections of cystic fibrosis patients

Preamble

The clinical hypermutator susceptibilities to three antibiotics, meropenem, tobramycin and ciprofloxacin (determined in Chapter 4), that displayed antibacterial properties against hypermutable PAOΔ*mutS* in 48-h static concentration time-kill assays (observed in Chapter 3) were initially considered for our choice of an antibiotic combination to combat these hypermutators. As the hypermutators were most frequently meropenem-susceptible, this antibiotic was chosen for evaluation. Preliminary static concentration time-kill screening was undertaken for meropenem in combination with other antibiotics with different mechanisms of action against clinical hypermutators in static concentration time-kill studies over 48 h. This screening found meropenem and ciprofloxacin to be most promising (data not shown). This Chapter addressed **Aim 4** by evaluating this promising, synergistic meropenem and ciprofloxacin combination using clinically relevant regimens against the well characterised, isogenic hypermutable PAOΔ*mutS* and non-hypermutable PAO1, and several clinical hypermutators (characterised in Chapter 5). The meropenem and ciprofloxacin combination was assessed in 72-h SCTK experiments and suppressed the emergence of resistance in both hypermutable and non-hypermutable *P. aeruginosa*. Subsequently, this result led to the first evaluation of this combination in the gold-standard *in vitro* dynamic hollow fibre infection model over 8 days with concentration-time profiles simulated for epithelial lining fluid as would be observed in CF patients. The application of MBM allowed the bacterial time-course to be described quantitatively. The results ultimately supported **Hypothesis 4**, suggesting the fluoroquinolone and β-lactam combination to be a highly promising option to combat hypermutable bacterial 'superbugs' present in the lungs of patients with CF.

Manuscript

Meropenem in combination with ciprofloxacin combats hypermutable *Pseudomonas aeruginosa* from respiratory infections of cystic fibrosis patients

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Running title: Meropenem with ciprofloxacin combats hypermutators

Key words: Fluoroquinolone; carbapenem; mechanism-based modeling; hypermutation; antibiotic resistance; pharmacokinetics / pharmacodynamics; combination therapy; epithelial lining fluid

Abstract

Hypermutable *Pseudomonas aeruginosa* are prevalent in chronic respiratory infections, have been associated with reduced lung function in cystic fibrosis (CF), and can become resistant to all antibiotics in monotherapy. This study aimed to evaluate the time-course of bacterial killing and resistance of ciprofloxacin and meropenem in combination against hypermutable and non-hypermutable *P. aeruginosa*. Static concentration time-kill experiments over 72h assessed meropenem and ciprofloxacin in mono- and combination therapy against PAO1 (non-hypermutable), PAOΔ*mutS* (hypermutable), and hypermutable isolates CW08, CW35 and CW44 obtained from CF patients with respiratory infections. Meropenem (1 or 2g q8h as 3h infusion and 3g/day as continuous infusion) and ciprofloxacin (400mg q8h as 1h infusion) in monotherapies and combinations were further evaluated in an 8-day hollow fiber infection model study (HFIM) against CW44. Concentration-time profiles in epithelial lining fluid representative of the pharmacokinetics in CF patients were simulated. Counts of total and resistant bacteria were determined and subjected to mechanism-based modeling (MBM). In the HFIM, all monotherapies resulted in rapid regrowth with resistance at 48h. The maximum daily doses of 6g meropenem ($T_{>MIC}$ 80-88%) and 1.2g ciprofloxacin (AUC/MIC of 176), both given intermittently, in monotherapy failed to supress regrowth and resulted in substantial emergence of resistance (≥7.6$\log_{10}$ CFU/mL resistant populations). However, these regimens in combination achieved synergistic killing and resistance suppression. MBM with subpopulation and mechanistic synergy yielded unbiased and precise curve fits. Thus, the combination of 6g/day meropenem plus ciprofloxacin is promising for future clinical evaluation against infections by susceptible hypermutable *P. aeruginosa*.

Introduction

Hypermutable *Pseudomonas aeruginosa* strains are a major concern in chronic respiratory infections of patients with cystic fibrosis (CF) due to reduced respiratory function and worsened patient outcomes (1-4). Hypermutable strains arise from mutations in DNA or deficient error repair systems which result in an increased mutation rate allowing the rapid development of resistance (2, 5-7). Monotherapy with currently available antibiotics has become ineffective against these hypermutable *P. aeruginosa* strains (2, 8). Therefore, the use of antibiotic combinations is vital against these difficult to treat infections by hypermutable *P. aeruginosa* in CF patients (9, 10). There is a dire need for safe and effective combinations that suppress the emergence of resistance related to hypermutable *P. aeruginosa*.

A combination of two antipseudomonal antibiotics such as ciprofloxacin and meropenem that have different dynamics of bacterial killing, and divergence in mechanisms of action and resistance, would appear worthy of consideration (11-14). This combination has been examined using the checkerboard method, MIC evaluation and 24-h static concentration time-kill (SCTK) assays against *P. aeruginosa* from diverse patient groups, with variable synergy outcomes (15-21). None of these studies employed a dynamic system to evaluate the combination of meropenem and ciprofloxacin, nor did they examine the time-course of resistant populations. Importantly, none of the aforementioned studies examined hypermutable isolates.

The main objective of the current investigation was to evaluate the combination of ciprofloxacin and meropenem against hypermutable and non-hypermutable *P. aeruginosa*, including isolates obtained from CF patients. We assessed both bacterial killing and resistance suppression with clinically relevant concentrations of ciprofloxacin and meropenem alone and in combination. Initially, we conducted SCTK studies against a range of reference and clinical

isolates. Subsequently, we simulated the human pharmacokinetics (PK) of each drug in the dynamic hollow fiber infection model (HFIM) and evaluated the antimicrobial effects of this combination against a clinical hypermutable *P aeruginosa* isolate. Pharmacokinetic/pharmacodynamic (PK/PD) mechanism-based modeling (MBM), investigations into ciprofloxacin front-loading and meropenem intermittent *versus* continuous infusion were incorporated in this work.

Results

Static-concentration time-kill studies. The MICs of meropenem and ciprofloxacin against the isolates used in SCTK are shown in **Table 1**. The PAO1 (non-hypermutable), PAOΔ*mutS* and CW44 were double-susceptible, the CW08 and CW35 were meropenem-susceptible and ciprofloxacin-resistant. Meropenem and ciprofloxacin monotherapies achieved ≤5.6 $\log_{10}$ CFU/mL bacterial killing against the non-hypermutable PAO1. Only the lowest 2.4 mg/L meropenem monotherapy exhibited regrowth of a resistant population (~7.2 $\log_{10}$ CFU/mL). Ciprofloxacin 0.6 and 1.2 mg/L failed to suppress resistant populations (~6.9-8.8 $\log_{10}$ CFU/mL) in monotherapy (**Fig. 1**). All combinations produced killing with no colonies from 48 h. Synergy (*i.e.* defined as an antibiotic combination resulting in ≥2 $\log_{10}$ of bacterial killing compared to the most active antibiotic in monotherapy) was observed for all 0.6 and 1.2 mg/L ciprofloxacin combinations (**Fig. 1**). Against the hypermutable PAOΔ*mutS*, the monotherapies achieved ≤4.3 $\log_{10}$ CFU/mL killing followed by regrowth to ≥8.0 $\log_{10}$ CFU/mL with resistant populations of ≥7.0 $\log_{10}$ CFU/mL. The combinations produced extensive bacterial killing of PAOΔ*mutS* with synergy achieved from 24-72 h onwards. Only the two lowest concentrations in combination had substantial regrowth to ~6.7 $\log_{10}$ CFU/mL, with a ciprofloxacin-resistant population (~5.1 $\log_{10}$

CFU/mL), but not for meropenem (**Fig. 1**). The hypermutable CW08 and CW35 strains were subject to treatment failure for all mono- and combination therapies with 2.4 and 4.8 mg/L meropenem. Synergy was only observed from 48 to 72 h for 12 mg/L meropenem combinations against CW08, and at 72 h for 12 mg/L meropenem with 3 mg/L ciprofloxacin against CW35 (**Fig. 1**).

Against the hypermutable CW44, the monotherapies achieved ≤3.3 $\log_{10}$ CFU/mL bacterial killing followed by substantial regrowth of susceptible and resistant populations to ≥8.5 $\log_{10}$ CFU/mL. Extensive bacterial killing of CW44 with synergy was observed for 4.8 and 12 mg/L meropenem combinations, however suppression of regrowth and resistant populations was only observed for the 12 mg/L meropenem with 1.2 and 3 mg/L ciprofloxacin combinations. MBM was performed to characterize the time-course of bacterial killing and regrowth for the meropenem and ciprofloxacin combination against this double-susceptible hypermutable isolate. Our MBM yielded unbiased and precise curve fits (**Fig. 1**, **S1** and **Table S1**). The coefficient of correlation for the observed *versus* individual fitted viable counts was 0.99. Subpopulation synergy alone was not sufficient to describe the time-course of bacterial killing and regrowth. The inclusion of mechanistic synergy (*i.e.* ciprofloxacin enhancing the antibacterial effects of meropenem) significantly improved the model performance. This synergy demonstrated in the SCTK and MBM prompted us to undertake a dynamic evaluation of this promising combination against CW44 in the HFIM.

The results of SCTK studies with the hypermutable PAOΔ*mutS*, CW08 and CW44 to evaluate a potential benefit of front-loading ciprofloxacin are presented in **Fig. S2-4**. Overall, our results showed that there was no great observable difference between the constant *versus* front-loaded ciprofloxacin in the SCTK.

Hollow fiber-infection model. Agreement between measured and targeted concentrations of meropenem and ciprofloxacin simulated profiles was on average within 10%. The results of the HFIM study with CW44 are presented in **Fig. 2**. The intermittent meropenem (1 and 2 g thrice-daily, 3 h infusion) and ciprofloxacin (standard regimen, 400 mg thrice daily, 1 h infusion) monotherapy treatments resulted in ~1.7-3.6 and ~4.1 $\log_{10}$ CFU/mL bacterial killing, respectively. This was followed by regrowth to ≥6.4 and ~8.6 $\log_{10}$ CFU/mL of populations resistant to meropenem and ciprofloxacin by 48 h, respectively. The meropenem 3 g standard daily dose as continuous infusion in monotherapy showed very limited bacterial killing (~0.4 $\log_{10}$ CFU/mL) with regrowth to ~7.7 $\log_{10}$ CFU/mL at 24 h with a meropenem-resistant population of ~4.7 $\log_{10}$ CFU/mL; by 48 h this meropenem-resistant population was ~6.4 $\log_{10}$ CFU/mL. The standard 3 g meropenem daily dose, intermittent and continuous infusion regimens in combination with the standard ciprofloxacin regimen provided similar bacterial responses. There was ~5.8 $\log_{10}$ CFU/mL bacterial killing followed by regrowth after 31 and 55 h; by 191 h there were ~5.5-6.7 $\log_{10}$ CFU/mL meropenem- and ~8.2-8.6 $\log_{10}$ CFU/mL ciprofloxacin-resistant populations. The 6 g maximum daily dose of meropenem given intermittently, simulated for two reported ELF: serum concentration ratios (30% and 63%) (22, 23), in combination with the standard ciprofloxacin regimen produced maximal bacterial killing and suppression of regrowth and resistance (**Fig. 2**).

The dynamic HFIM data further informed our MBM (with very similar structure to the SCTK MBM) yielding unbiased and precise curve fits for all regimens (**Fig. 2**, **S1** and **Table 2**). The coefficient of correlation was 0.99 for the observed *versus* individual fitted viable counts.

Discussion

We sought to evaluate the antibacterial effects of the meropenem and ciprofloxacin combination against hypermutable and non-hypermutable *P. aeruginosa*, including isolates from CF patients. In this work we used SCTK and MBM to initially evaluate bacterial killing and resistance suppression with clinically relevant concentrations of ciprofloxacin and meropenem in mono- and combination therapy. The meropenem and ciprofloxacin combination was promising against hypermutable and non-hypermutable strains in 72 h SCTK studies. Previous studies over 24 h also found both susceptible and resistant isolates exhibited synergy (18, 21). We further evaluated this combination in the dynamic HFIM simulating human PK, whilst considering ELF penetration (representative of CF patients with a lack of an immune system response). Our findings indicated that monotherapy with meropenem or ciprofloxacin (even with the 6 and 1.2 g maximum daily doses, respectively) was not sufficient to suppress bacterial regrowth and resistance. However, these intermittent regimens in combination demonstrated synergistic bacterial killing and suppression of resistance emergence.

For ciprofloxacin, bacterial killing and clinical success have been found to best correlate with the free area under the plasma concentration-time curve (*f*AUC) and the free peak concentration (fC_{max}) divided by the MIC (24-27). In acutely ill patients, an AUC/MIC of 125 (*f*AUC/MIC of 87.5) was correlated with clinical success against bacterial infections (28). However, in hospitalized patients infected with strains having MICs of ≥1 mg/L this AUC/MIC target was often not reached (29). In our previous work, we found that the shape of the exposure profile associated with a given *f*AUC is critical in ciprofloxacin-resistance suppression, with short-course, high-concentration exposure minimizing resistance (30). This was in agreement with other studies which found that higher fluoroquinolone peak concentrations (C_{max}/MIC ratios

>10/1) prevented resistance emergence (31, 32). In the present study using hypermutable *P. aeruginosa*, the AUC/MIC for ciprofloxacin in ELF was 176 with C_{max}/MIC of 14.7, both well exceeding the PK/PD targets described above, but monotherapy was not successful.

Administration of meropenem usually requires maximization of the fraction of the dosing interval that the unbound concentration remains above the MIC of the strain ($fT_{>MIC}$). It has been described that at least 40% $fT_{>MIC}$ will usually provide optimal bactericidal activity (27, 33), although studies demonstrated that 100% $fT_{>MIC}$ was required in serious bacterial infections to suppress regrowth (34-36). Minimum concentrations (fC_{min}) ≥4x MIC, or >5 to 6×MIC, have further been established for the suppression of meropenem resistance emergence (37-40). However, the use of synergistic combination therapy does not usually require these PK/PD targets to be as high (37-39).

In the present study representing ELF concentrations, all intermittent infusion meropenem regimens had a $T_{>MIC}$ between 69% and 88% with a C_{min}/MIC of ≤0.4, whilst 3 g meropenem daily as continuous infusion achieved 100% $T_{>MIC}$ with a C_{min}/MIC of 4.8 mg/L (**Table 3**). As anticipated, all meropenem intermittent infusion regimens in monotherapy resulted in the emergence of resistance. The intermittent infusion of 6 g meropenem in combination with ciprofloxacin achieved suppression of the emergence of resistance despite not reaching the recommended PK/PD targets. In contrast, the 3 g per day continuous infusion failed to suppress regrowth from 24 h in both mono- and combination therapy with ciprofloxacin. It achieved the 100% $T_{>MIC}$ target, but was borderline regarding the recommended C_{min}/MIC targets. The meropenem Etest MIC of 0.5 mg/L for CW44 (Table 1) reflects the susceptibility of the predominant bacterial population. However, resistant mutant subpopulations (RMS) are frequently present in hypermutable isolates (41), and we have observed RMS for CW44 against

meropenem. This is further supported by the meropenem agar MIC using the CLSI method, which was 4 mg/L for CW44. Therefore, the $T_{>MIC}$ and C_{min}/MIC values would likely be decreased when considering these subpopulations.

The developed MBM well described the antibacterial effects of meropenem and ciprofloxacin in mono- and combination therapy against CW44. The data required three pre-existing bacterial populations: double susceptible; meropenem-resistant, ciprofloxacin-intermediate; and meropenem-intermediate, ciprofloxacin-resistant. Both subpopulation and mechanistic synergy were required to describe the antibacterial effects of the combination, similar to a previous study with MBM for an aminoglycoside and carbapenem combination (42). Subpopulation synergy, where meropenem killed ciprofloxacin-resistant bacteria and *vice versa*, was based on meropenem and ciprofloxacin having different resistance mechanisms; such as target site mutations (ciprofloxacin), AmpC β-lactamase overexpression (meropenem) and inactivation of outer membrane porin *oprD* (meropenem) (11-14). Mechanistic synergy involved enhanced bacterial killing by meropenem in the presence of ciprofloxacin and was likely due to ciprofloxacin permeabilizing the outer membrane of *P. aeruginosa* to meropenem (43). Additionally, this mechanistic synergy would be further owing to ciprofloxacin minimizing AmpC expression that has been found to be enhanced with exposure to meropenem alone (44).

We are not aware of any previous dynamic studies that evaluated the antibacterial effects of the meropenem with ciprofloxacin combination for CF patients with hypermutable *P. aeruginosa* in the HFIM. Furthermore, the extent and time-course of bacterial killing and regrowth for this combination regimen have not been characterized by an MBM. In conclusion, this study indicated that meropenem in combination with ciprofloxacin is a promising synergistic combination against hypermutable *P. aeruginosa* when simulating CF patient PK in ELF. MBM

on this combination adequately characterized the synergistic bacterial killing and resistance suppression *via* both subpopulation and mechanistic synergy. Future studies are warranted to optimize this meropenem and ciprofloxacin combination to further enhance resistance suppression, which is vital as the next step to translate this data for the use in clinical studies.

Materials and methods

Bacterial strains and media. The non-hypermutable *P. aeruginosa* PAO1 wild-type reference strain, the hypermutable *P. aeruginosa* PAOΔ*mutS* strain (PAO1 with the *mutS* gene knocked out) (45) and three hypermutable *P. aeruginosa* isolates from respiratory infections of cystic fibrosis patients (CW08, CW35 and CW44; MICs in **Table 1**) were evaluated. All studies were performed using cation-adjusted Mueller Hinton broth (CAMHB; containing 20 to 25 mg/L Ca^{2+} and 10 to 12.5 mg/L Mg^{2+}; BD, North Ryde, NSW, Australia) and total viable counting was performed on antibiotic-free cation-adjusted Mueller Hinton agar (CAMHA; containing 25 mg/L Ca^{2+} and 12.5 mg/L Mg^{2+}; Medium Preparation Unit, University of Melbourne, Parkville, Australia) plates. Viable counting of resistant populations was carried out on antibiotic-containing CAMHA (BD, North Ryde, NSW, Australia) plates, supplemented with 1.25 mg/L ciprofloxacin (Sigma-Aldrich, Sydney, Australia) or 10 mg/L meropenem (Hospira, Melbourne, Australia). The antibiotic stock solutions were prepared in Milli-Q water and subsequently filter-sterilized using a 0.22 μm PVDF syringe filter (Merck Millipore, Cork, Ireland).

Static concentration time-kill experiments. To evaluate bacterial killing and resistance suppression of the ciprofloxacin and meropenem combination, SCTK studies were conducted over 72 h against all five strains. Clinically representative lung epithelial lining fluid (ELF) concentrations of 0.6, 1.2 and 3 mg/L ciprofloxacin, and 2.4, 4.8 and 12 mg/L meropenem were

used in mono- and combination therapy (22, 46, 47). The 1.2 and 3 mg/L ciprofloxacin was representative of the average and peak concentrations, respectively, of the ciprofloxacin recommended daily dose (1200 mg) in CF patients, assuming 85% ELF penetration (48, 49). The 2.4 mg/L meropenem was representative of the average ELF concentration with the recommended daily dose (3 g), whilst 4.8 and 12 mg/L meropenem represented the average and peak ELF concentrations, respectively, with the highest daily dose (6 g) in CF patients, assuming 30% ELF penetration (22). Further evaluation of ciprofloxacin was performed by assessing 'front-loading' ciprofloxacin (3.3 mg/L for 1.5 h followed by 1 mg/L) and 8 and 16 mg/L meropenem, alone and combined, against hypermutable PAOΔ*mutS*, CW08, and CW44 in 72 h SCTK studies (methods are in **Table S2**).

In preparation for SCTK studies an overnight culture was grown in sterile CAMHB incubated at 35°C in a shaking waterbath. The optical density of the bacterial suspension was measured using a spectrophotometer and appropriately diluted to achieve the targeted initial inoculum of ~$10^{7.4}$ CFU/mL in 15 mL CAMHB. These bacterial suspensions were incubated at 35°C in a shaking waterbath and the appropriate volumes of antibiotic stock solution were added at the initiation of the time-kill experiments. At 24 and 48 h in all SCTK studies, 100% re-dosing of both antibiotics after a centrifugation and re-suspension step was carried out; an additional 30% of meropenem was supplemented at 6 and 30 h to offset thermal degradation (50, 51). An antibiotic-free growth control was included for all tested isolates in all SCTK studies.

Samples for viable counts of the total population were collected at 0 (prior to antibiotic dosing), 1.5, 3, 6, 24, 48 and 72 h; the 72 h viable count samples were also used to determine meropenem- or ciprofloxacin-resistant populations. Samples were washed twice with sterile saline *via* centrifugation and re-suspended to minimize antibiotic carryover. Viable counts were

determined by plating 100 μL (or 200 μL for resistant populations) of undiluted or 10-fold serially diluted (in saline) bacterial suspensions on CAMHA. This plating method yielded a limit of counting of 1.0 $\log_{10}$ CFU/mL (or 0.7 $\log_{10}$ CFU/mL for resistant populations). Agar plates were incubated at 35°C for 48 - 72 h.

Dynamic hollow-fiber infection model. The hypermutable *P. aeruginosa* CW44 strain was used in the dynamic hollow-fiber infection model (HFIM) to further assess the meropenem and ciprofloxacin combination. Ciprofloxacin and meropenem regimens for CF patients were simulated alone and in combination in the HFIM over 8 days. The ciprofloxacin standard clinical regimen of 400 mg thrice daily administered *via* a 1-h infusion was used. The meropenem regimens included the standard dose of 1 g thrice daily and maximum clinical dose of 2 g thrice daily, both administered *via* a 3-h infusion (52). A meropenem regimen of 3 g (standard daily dose) given daily *via* continuous infusion was also evaluated. The simulated PK profiles in the HFIM were based on the antibiotic concentrations over time that would be expected in the ELF of CF patients following the respective dosage regimens. These expected ELF concentration-time profiles were determined by *in silico* simulations using Berkeley Madonna (version 8.3.18, **Table 3**), based on published clinical PK studies in CF patients and reports on the ELF penetration of ciprofloxacin (85%) and meropenem (22, 23, 48, 49). For meropenem both 30 and 63% penetration were simulated based on the relevant literature reports. The simulated half-lives were 2.9 h for ciprofloxacin and 0.8 h for meropenem (46, 47, 53). The peak ELF concentrations (meropenem concentrations based on 30% ELF penetration (22)) and relevant PK/PD data are displayed in **Table 3**. The highest approved (2g q8h) meropenem regimen was also simulated with the higher ELF penetration (63%) that was reported recently (23); with a peak ELF concentration of 24 mg/L. A loading dose was given as a bolus in the HFIM for each

ciprofloxacin treatment in mono- and combination therapy, as well as for the 3 g per day meropenem continuous infusion treatments, to simulate steady-state. A no-treatment growth control was included.

The HFIM study was conducted utilizing cellulosic cartridges (C3008-1, FiberCell Systems Inc., Frederick, MD, USA) in a humidified incubator at 36°C. An overnight bacterial suspension was prepared in CAMHB at 36°C. The optical density of the overnight culture was measured spectrophotometrically and was diluted to achieve the targeted initial inoculum of ~$10^{7.4}$ CFU/mL. HFIM cartridges were injected with 17 mL of this bacterial suspension. Viable count samples were collected aseptically at 0, 3.5, 7, 11.5, 23, 27.5, 31, 47, 51.5, 55, 71, 95, 119, 143, 167 and 191 h, washed *via* centrifugation and re-suspended to remove antibiotic, as described above, and plated on antibiotic-free CAMHA. Resistant populations were quantified on antibiotic-containing agar at 0, 23, 47, 71, 95, 119, 143, 167 and 191 h.

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) analysis. Samples of 1 mL were periodically collected in duplicate from the central reservoir outflow of the HFIM and immediately stored at -80ºC until assayed by LC-MS/MS to determine the concentrations of meropenem and ciprofloxacin. A Waters Acquity H-class UHPLC system coupled with Waters Xevo TQS triple quadrupole mass spectrometer was utilized. Data acquisition was performed using MassLynx version 4.1 software. Four levels of analytical QC samples were analyzed in duplicate and both analytes were within 15%.

Mechanism-based modeling of bacterial killing and resistance. MBM was performed for the SCTK (CW44 isolate only) and HFIM data utilizing S-ADAPT (version 1.57, importance sampling algorithm, pmethod=4) (54). The life-cycle growth model was used to describe the growth and replication of *P. aeruginosa* (55-57). We evaluated subpopulation synergy (*i.e.*

meropenem killing the bacteria resistant to ciprofloxacin and *vice versa*) and mechanistic synergy (*i.e.* ciprofloxacin enhancing the killing by meropenem against one or multiple bacterial populations) (42, 56, 58). A diagram of the model structure is shown in **Fig. 3**. The differential equations are available in the online supplement. Competing models were evaluated based on the biological plausibility of the parameter estimates, the S-ADAPT objective function value (-1×log-likelihood), standard diagnostic plots, the coefficient of correlation and visual predictive checks (59-62). The SCTK MBM was adapted for the HFIM data.

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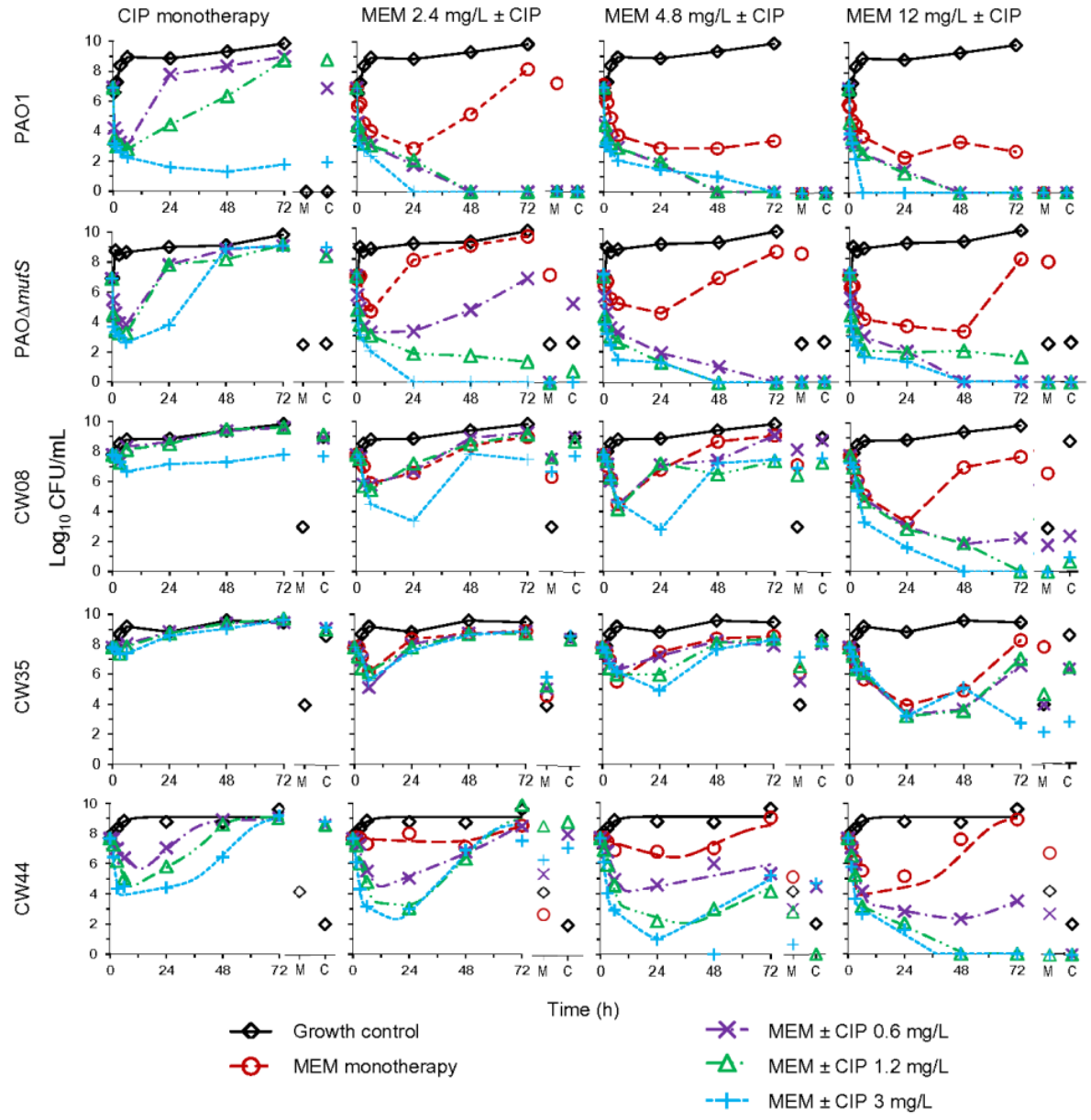

Fig. 1: The effect over 72 h of ciprofloxacin monotherapy (column 1) and meropenem with ciprofloxacin combinations (columns 2-4) against non-hypermutable *P. aeruginosa* PAO1 and hypermutable *P. aeruginosa* PAOΔ*mutS*, CW08, CW35 and CW44 (top to bottom rows, respectively) in static concentration time-kill studies on the total bacterial population. The resistant bacterial populations were quantified on 10 mg/L meropenem (M) and 1.25 mg/L ciprofloxacin (C) containing agar plates at 72 h. The CW44 bacterial time-course are shown as observed viable counts (symbols) and individual predicted profiles (lines in corresponding colours) from mechanism-based modeling.

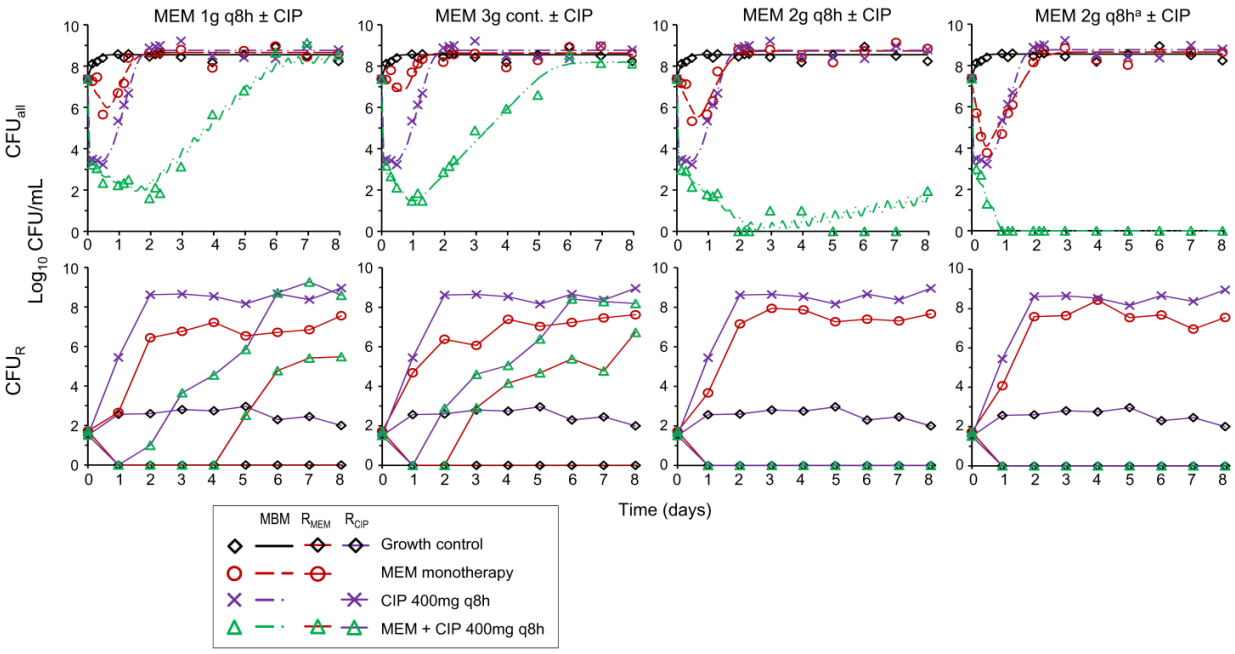

Fig. 2: The antibacterial effect of meropenem and ciprofloxacin regimens in mono- and combination therapy against hypermutable *P. aeruginosa* CW44 in the dynamic hollow fiber infection model over 8 days. The meropenem regimens were: 1 g thrice daily as 3 h infusion, 3 g daily as continuous infusion and 2 g thrice daily as 3 h infusion, all representative of 30% ELF penetration, and 2 g thrice daily as 3 h infusion representative of 63%[a] ELF penetration, left to right columns, respectively. The ciprofloxacin regimen was 400 mg thrice daily as 1 h infusion. The top row shows the total bacterial population (CFU_{ALL}; observed viable counts, symbols, and individual predicted profiles of the mechanism-based modeling, MBM, lines in corresponding colours). The bottom row shows the resistant bacterial populations (CFU_R) of meropenem and ciprofloxacin in mono- and combination therapy against CW44 quantified on 10 mg/L meropenem (R_{MEM}, red lines) and 1.25 mg/L ciprofloxacin (R_{CIP}, purple lines) containing agar plates.

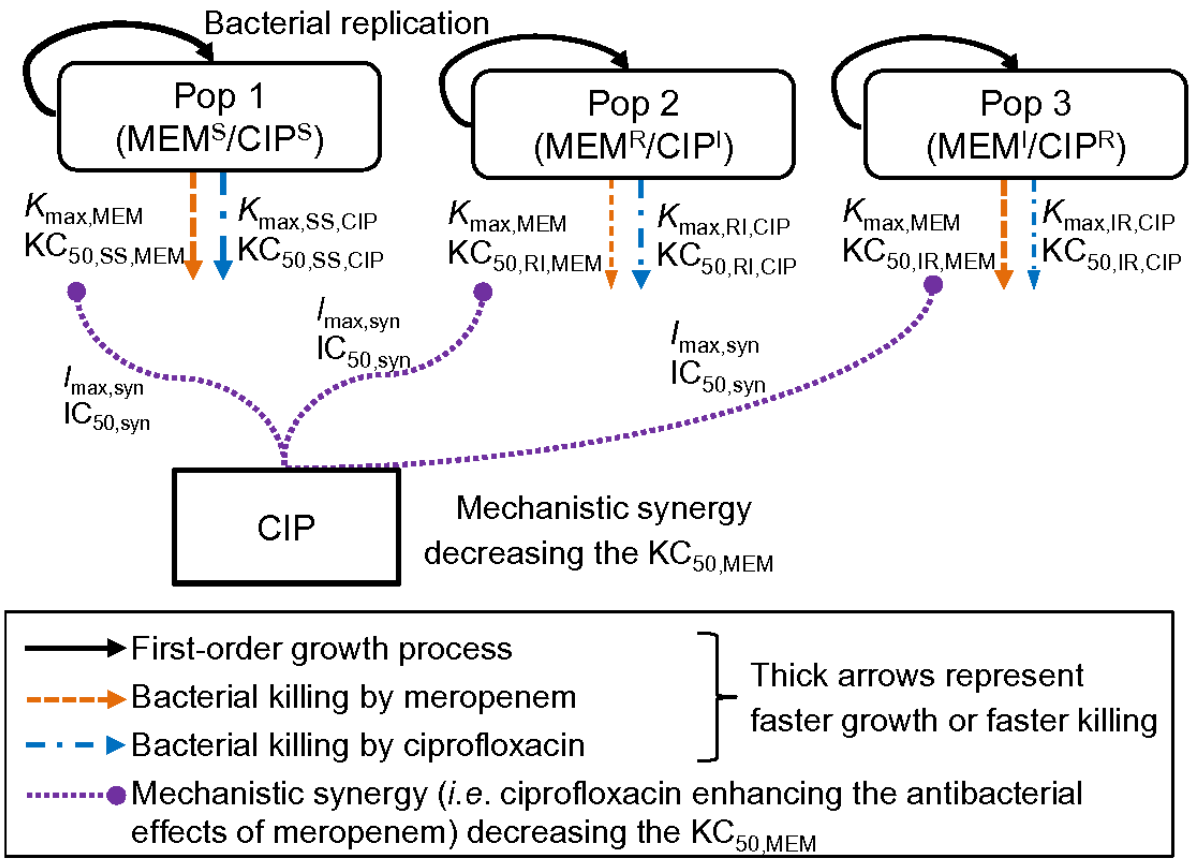

Fig. 3: The mechanism-based model for bacterial growth and killing by meropenem and ciprofloxacin in mono- and combination therapy. The first population is double-susceptible, *i.e.* was susceptible to both meropenem and ciprofloxacin (MEM^S/CIP^S). The other two populations not shown were meropenem-resistant and ciprofloxacin-intermediate (MEM^R/CIP^I) or meropenem-intermediate and ciprofloxacin-resistant (MEM^I/CIP^R). A life-cycle growth model describes the underlying biology of bacterial replication in two states for each population. The maximum killing rate constants (K_{max}) and related antibiotic concentrations causing 50% of K_{max} (KC_{50}), along with all parameter estimates are described in **Table S1** (SCTK data) and **Table 2** (HFIM data). Mechanistic synergy (*i.e.* ciprofloxacin enhancing the effect of meropenem) was included for all populations.

Table 1: The MICs of meropenem (MEM) and ciprofloxacin (CIP) for the non-hypermutable *P. aeruginosa* PAO1 and hypermutable *P. aeruginosa* PAOΔ*mutS*, CW08, CW35 and CW44 strains, obtained using Etest (bioMérieux, North Ryde, Australia). MICs for PAO1 and PAOΔ*mutS* were previously reported (41).

Strain	MIC_{MEM} (mg/L)	MIC_{CIP} (mg/L)
PAO1	0.38	0.064
PAOΔ*mutS*	0.5	0.064
CW08	1.5	4
CW35	0.5	3
CW44	0.5	0.19

Table 2: Population mean (SE %) parameter estimates for the mechanism-based model (MBM) evaluation of the dynamic hollow fiber infection model (HFIM) experiments assessing the meropenem with ciprofloxacin combination against hypermutable *P. aeruginosa* CW44. All parameters were required for the MBM to achieve unbiased and precise curve fits.

Parameter	Symbol (unit)	Population mean value (SE[%])
Bacterial growth and subpopulations		
Initial inoculum	$Log_{10}CFU_0$	7.43 (2.33%)
Maximum population size	$Log_{10}CFU_{max}$	8.71 (2.03%)
Replication rate constant	k_{21} (h^{-1})	50 (fixed)[a]
Mean generation time (MGT)		
MEM^S/CIP^S	$k_{12,SS}$ (min^{-1})	97.6 (fixed)
MEM^R/CIP^I	$k_{12,RI}$ (min^{-1})	162 (6.23%)
MEM^I/CIP^R	$k_{12,IR}$ (min^{-1})	162 (6.23%)
Log_{10} mutation frequencies		
MEM	$Log_{10}MF_{MEM}$	-4.28 (5.48%)
CIP	$Log_{10}MF_{CIP}$	-7.96 (11.5%)
Killing by MEM		
Maximum killing rate constant	$K_{max,MEM}$ (h^{-1})	0.907 (18.9%)
MEM concn causing 50% of $K_{max,MEM}$		
MEM^S/CIP^S	$KC_{50,SS,MEM}$ (mg/L)	0.105 (66.2%)
MEM^R/CIP^I	$KC_{50,RI,MEM}$ (mg/L)	241 (10.4%)
MEM^I/CIP^R	$KC_{50,IR,MEM}$ (mg/L)	4.79 (30.4%)
Hill coefficient for MEM	$HILL_{MEM}$	2.38 (10.1%)
Killing by CIP		
Maximum killing rate constant		
MEM^S/CIP^S	$K_{max,SS,CIP}$ (h^{-1})	6.65 (37.2%)
MEM^R/CIP^I	$K_{max,RI,CIP}$ (h^{-1})	1.14 (19.0%)
MEM^I/CIP^R	$K_{max,IR,CIP}$ (h^{-1})	0.0544 (63.6%)
CIP concn causing 50% of $K_{max,CIP}$		

MEM^S/CIP^S	$KC_{50,SS,CIP}$ (mg/L)	0.00918 (64.1%)
MEM^R/CIP^I	$KC_{50,RI,CIP}$ (mg/L)	1.40 (26.3%)
MEM^I/CIP^R	$KC_{50,IR,CIP}$ (mg/L)	25.7 (62.9%)
Mechanistic synergy		
Maximum fractional decrease of $KC_{50,MEM}$ *via* mechanistic synergy	$I_{max,syn}$	0.528 (0.399-0.653)[b]
CIP concn causing 50% of $I_{max,syn}$	$IC_{50,syn}$ (mg/L)	4.43 (24.0%)
SD of residual error on log_{10} scale	SD_{CFU}	0.341 (7.34%)

[a] Bacterial replication was assumed to be fast as previously described (63).

[b] 5^{th} – 95^{th} percentile

MEM: meropenem; CIP: ciprofloxacin; concn: concentrations

Table 3: The clinically representative epithelial lining fluid (ELF) concentrations and relevant pharmacokinetic / pharmacodynamic (PK/PD) parameters for meropenem (MEM, representative of 30% ELF penetration and 63%[a]) and ciprofloxacin (CIP) used alone and combined against hypermutable *P. aeruginosa* CW44 in the dynamic hollow fiber infection model.

Antibiotic	Regimen	PK/PD	C_{max} (mg/L)	C_{min} (mg/L)
CIP	400mg q8h (1h inf.)	AUC/MIC: 176	2.8	0.53
MEM	1g q8h (3h inf.)	$T_{>MIC}$ 69.3%	6	0.053
MEM	2g q8h (3h inf.)	$T_{>MIC}$ 80% (88%[a])	12	0.105
MEM	3g/day cont. inf.	$T_{>MIC}$ 100%	2.4	2.40

Supplementary material

Table S1: Population mean (SE %) parameter estimates for the mechanism-based model (MBM) evaluation of static concentration time-kill (SCTK) experiments assessing the meropenem with ciprofloxacin combination against hypermutable *P. aeruginosa* CW44. All parameters were required for the MBM to achieve unbiased and precise curve fits.

Parameter	Symbol (unit)	Population mean value (SE[%])
Bacterial growth and subpopulations		
Initial inoculum	$Log_{10}CFU_0$	7.77 (2.8%)
Maximum population size	$Log_{10}CFU_{max}$	9.14 (1.1%)
Replication rate constant	k_{21} (h^{-1})	50 (fixed)[a]
Mean generation time (MGT)		
MEM^S/CIP^S	$k_{12,SS}$ (min^{-1})	97 (fixed)
MEM^R/CIP^I	$k_{12,RI}$ (min^{-1})	158 (3.7%)
MEM^I/CIP^R	$k_{12,IR}$ (min^{-1})	129 (4.9%)
Log_{10} mutation frequencies		
MEM	$Log_{10}MF_{MEM}$	-4.15 (3.2%)
CIP	$Log_{10}MF_{CIP}$	-8.90 (2.9%)
Killing by MEM		
Maximum killing rate constant	-	-
MEM^S/CIP^S	$K_{max,SS,MEM}$ (h^{-1})	1.11 (22.4%)
MEM^R/CIP^I	$K_{max,RI,MEM}$ (h^{-1})	0.460 (24.5%)
MEM^I/CIP^R	$K_{max,IR,MEM}$ (h^{-1})	0.297 (45.5%)
MEM concn causing 50% of $K_{max,MEM}$		
MEM^S/CIP^S	$KC_{50,SS,MEM}$ (mg/L)	1.56 (13.1%)
MEM^R/CIP^I	$KC_{50,RI,MEM}$ (mg/L)	7.26 (9.7%)
MEM^I/CIP^R	$KC_{50,IR,MEM}$ (mg/L)	1.99 (25.4%)
Hill coefficient for MEM	$HILL_{MEM}$	0.663 (22.5%)
Killing by CIP		
Maximum killing rate constant		

MEM^S/CIP^S	$K_{max,SS,CIP}$ (h^{-1})	5.92 (18.9%)
MEM^R/CIP^I	$K_{max,RI,CIP}$ (h^{-1})	1.02 (26%)
MEM^I/CIP^R	$K_{max,IR,CIP}$ (h^{-1})	0.210 (29.1%)
CIP concn causing 50% of $K_{max,CIP}$		
MEM^S/CIP^S	$KC_{50,SS,CIP}$ (mg/L)	3.62 (10.6%)
MEM^R/CIP^I	$KC_{50,RI,CIP}$ (mg/L)	5.60 (15.9%)
MEM^I/CIP^R	$KC_{50,IR,CIP}$ (mg/L)	21.8 (10.6%)
Mechanistic synergy		
Maximum fractional decrease of $KC_{50,MEM}$ *via* mechanistic synergy	$I_{max,syn}$	0.660 (0.603-0.713)[b]
CIP concn causing 50% of $I_{max,syn}$	$IC_{50,syn}$ (mg/L)	0.786 (18.6%)
SD of residual error on log_{10} scale	SD_{CFU}	0.343 (11.4%)

[a] Bacterial replication was assumed to be fast as previously described (1).
[b] 5^{th} – 95^{th} percentile
MEM: meropenem; CIP: ciprofloxacin; concn: concentrations

Table S2: The clinically representative epithelial lining fluid (ELF) concentrations of meropenem (MEM) with constant and front-loaded ciprofloxacin (CIP) used alone and combined against hypermutable *P. aeruginosa* PAOΔ*mutS*, CW08 and CW44 strains in 72 h SCTK experiments. Less susceptible populations were determined on 5 mg/L meropenem- and 2.5 mg/L ciprofloxacin-containing agar plates at 24, 48 and 72 h.

Strain	MEM	CIP front-load†
PAOΔ*mutS*	8 & 16 mg/L	3.3 mg/L for 1 h or 2.1 mg/L for 2 h then 1mg/L
CW08	8 mg/L	3.3 mg/L for 1.5 h then 1 mg/L
CW44	8 & 16 mg/L	3.3 mg/L for 1.5 h then 1 mg/L

† Front-loading was achieved by dosing ciprofloxacin at 0 h then removing after the indicated specific time *via* a centrifugation and re-suspension in CAMHB containing 1 mg/L ciprofloxacin (or antibiotic-free CAMHB for front-loading controls). Front-loading controls were included for all tested treatments.

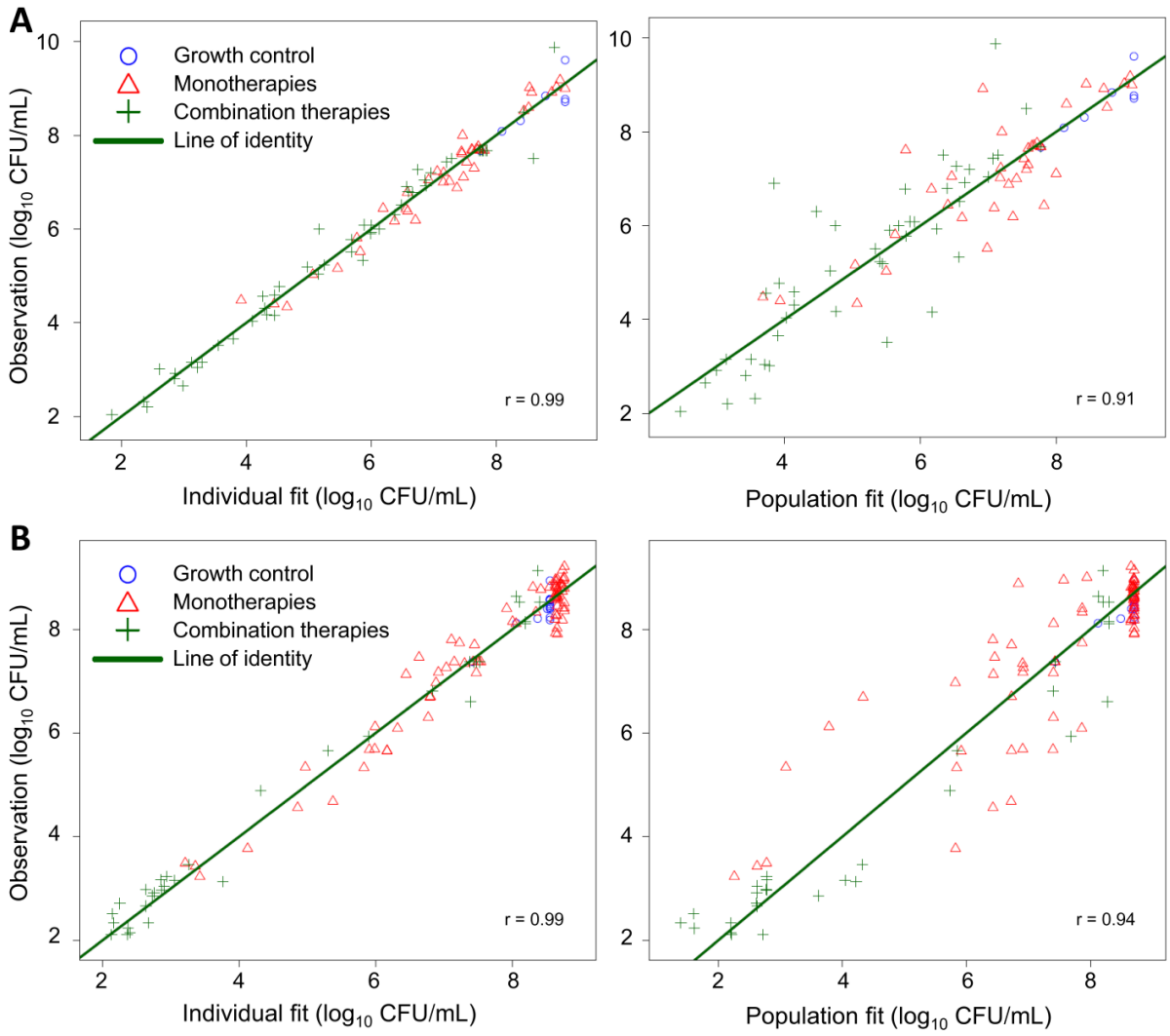

Fig. S1: Observed vs. individual (left) and population (right) fitted viable counts for meropenem and ciprofloxacin monotherapies and combinations against hypermutable *P. aeruginosa* CW44 in 72 h static concentration time-kill studies (**A**) and an 8-day dynamic hollow fiber infection model (**B**).

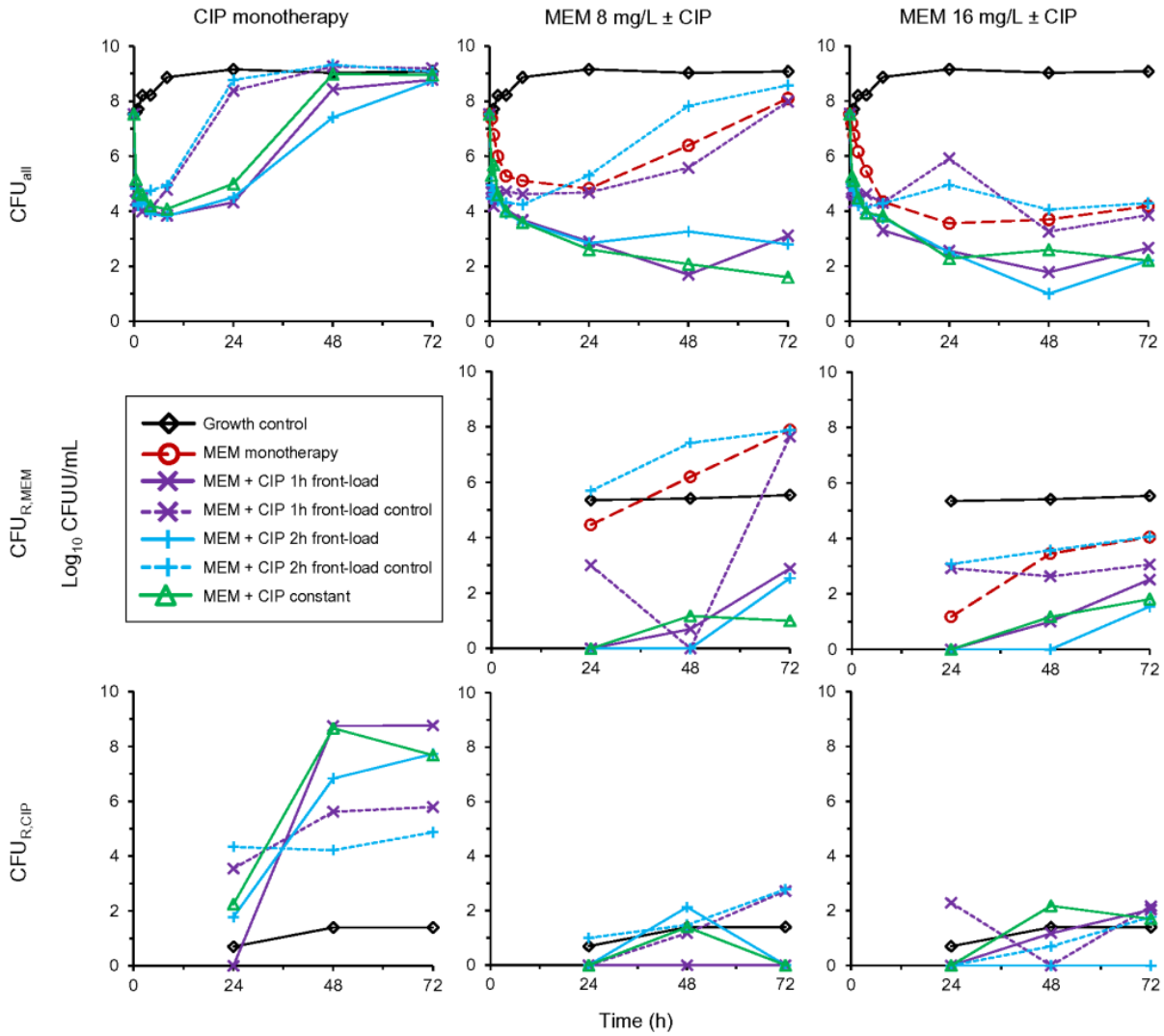

Fig. S2: The effect over 72 h of ciprofloxacin (front-loaded *vs.* constant; methods in **Table S2**) monotherapy (left column) and meropenem with ciprofloxacin combinations (middle and right columns) against hypermutable *P. aeruginosa* PAOΔ*mutS* in static concentration time-kill studies. The total (top row) and non-susceptible bacterial populations quantified on 5.0 mg/L meropenem-containing agar plates (middle row) and 2.5 mg/L ciprofloxacin-containing agar plates, (bottom row) are displayed. Meropenem and ciprofloxacin monotherapies led to extensive emergence of resistance, the combination resulted in more bacterial killing and resistance suppression although no distinguishable difference was observed between front-loading and constant ciprofloxacin.

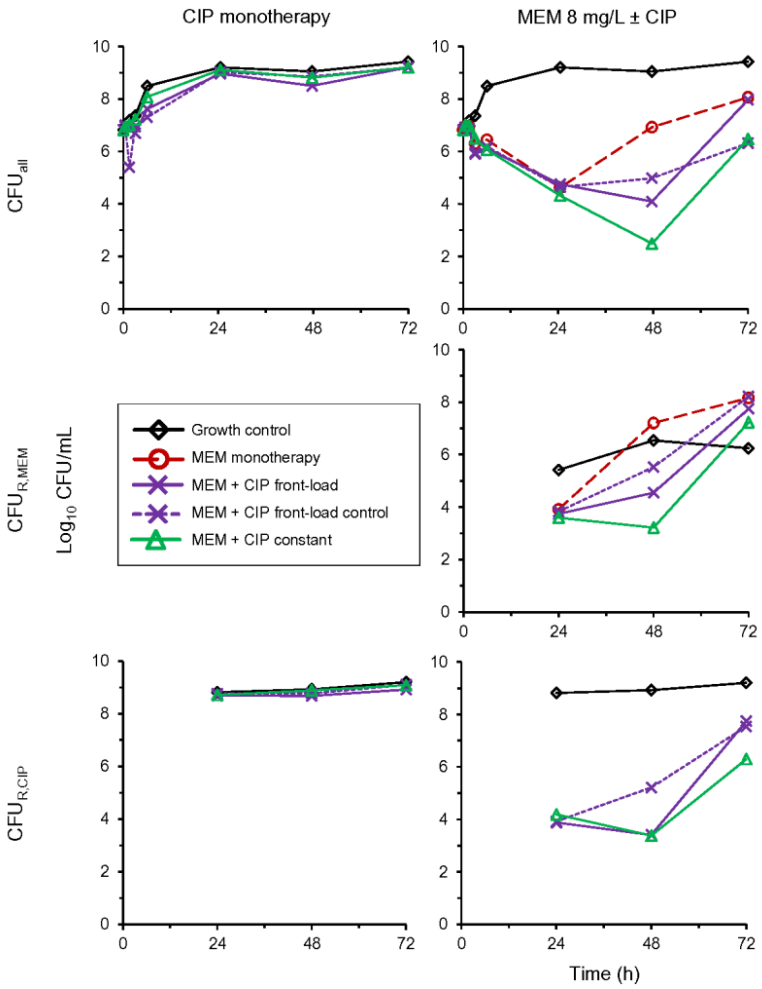

Fig. S3: The effect over 72 h of ciprofloxacin (front-loaded *vs.* static; methods in **Table S2**) monotherapy (left column) and meropenem with ciprofloxacin combinations (right column) against hypermutable *P. aeruginosa* CW08 in static concentration time-kill studies. The total (top row) and non-susceptible bacterial populations quantified on 5.0 mg/L meropenem-containing agar plates (middle row) and 2.5 mg/L ciprofloxacin-containing agar plates (bottom row) are displayed. Meropenem and ciprofloxacin monotherapies resulted in limited to no bacterial killing with regrowth and replacement by non-susceptible populations. The combination produced bacterial killing followed closely with regrowth of non-susceptible populations.

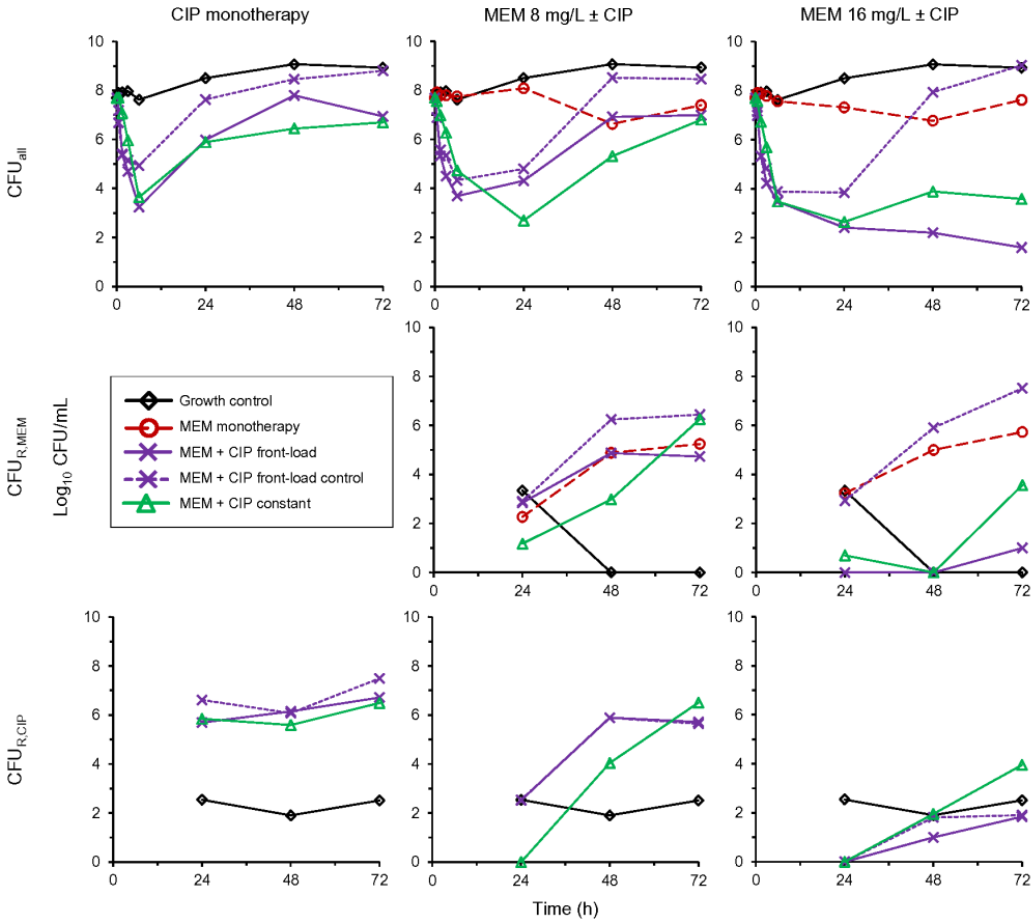

Fig. S4: The effect over 72 h of ciprofloxacin (front-loaded *vs*. static; methods in **Table S2**) monotherapy (left column) and meropenem with ciprofloxacin combinations (middle and right columns) against hypermutable *P. aeruginosa* CW44 in static concentration time-kill studies. The total (top row) and non-susceptible bacterial populations quantified on 5.0 mg/L meropenem-containing agar plates (middle row) and 2.5 mg/L ciprofloxacin-containing agar plates (bottom row) are displayed. Meropenem and ciprofloxacin monotherapies led to extensive emergence of resistance. The combination resulted in more prevention of regrowth although resistance was not suppressed. Furthermore differences observed between front-loading and static ciprofloxacin were limited.

Mechanism-based population PK/PD modeling

For isolate CW44, the data from the SCTK and HFIM studies were subjected separately to mechanism-based pharmacokinetic/pharmacodynamic (PK/PD) modeling (MBM) to quantitatively describe the time-course of the total population against ciprofloxacin and meropenem alone and combined. MBM utilized S-ADAPT (version 1.57) facilitated by SADAPT-TRAN with the importance sampling algorithm (pmethod=4) (2). A coefficient of variation of 15% during the end of estimation allowed the between-curve variability of the parameters to be fixed (3). Competing models were evaluated based on the S-ADAPT objective function value (-1×log-likelihood), visual predictive checks, standard diagnostic plots, the biological plausibility of the parameter estimates and the coefficient of correlation (4-6).

The life-cycle growth model was utilized to represent the growth and replication of *P. aeruginosa* (7-9). A diagram of the model structure is shown in **Fig. 3**. The proposed model for combinations of meropenem and ciprofloxacin comprised three pre-existing bacterial subpopulations; including double-susceptible (SS), meropenem-resistant and ciprofloxacin-intermediate (RI), and meropenem-intermediate and ciprofloxacin-resistant (IR) populations. Two bacterial states (*i.e.* compartments) for each of these subpopulations were described; including bacteria preparing for replication in state 1 and those immediately before replication in state 2 (8-11). The total bacterial population (CFU_{All}) was:

$$CFU_{All} = CFU_{SS1} + CFU_{SS2} + CFU_{RI1} + CFU_{RI2} + CFU_{IR1} + CFU_{IR2} \quad (1)$$

We used a direct killing process (12, 13) for both meropenem and ciprofloxacin. The equation for the double susceptible population in state 1 (CFU_{SS1}) was:

$$\frac{d(CFU_{SS1})}{dt} = 2 \cdot PLAT \cdot k_{21} \cdot CFU_{SS2} - k_{12,SS} \cdot CFU_{SS1} - \left(\frac{K_{max,SS,MEM} \cdot C_{MEM}^{Hill_{MEM}}}{C_{MEM}^{Hill_{MEM}} + (SYN \cdot (KC_{50,SS,MEM}^{Hill_{MEM}}))} + \frac{K_{max,SS,CIP} \cdot C_{CIP}}{C_{CIP} + KC_{50,SS,CIP}} \right) \cdot CFU_{SS1} \quad (2)$$

The double-susceptible population in state 2 (CFU_{SS2}) was described as:

$$\frac{d(CFU_{SS2})}{dt} = -k_{21} \cdot CFU_{SS2} + k_{12,SS} \cdot CFU_{SS1} - \left(\frac{K_{max,SS,MEM} \cdot C_{MEM}^{Hill_{MEM}}}{C_{MEM}^{Hill_{MEM}} + (SYN \cdot (KC_{50,SS,MEM}^{Hill_{MEM}}))} + \frac{K_{max,SS,CIP} \cdot C_{CIP}}{C_{CIP} + KC_{50,SS,CIP}} \right) \cdot CFU_{SS2} \quad (3)$$

The differential equations for the other two populations (RI and IR) were similar but included different estimates for K_{max}, KC_{50} and k_{12} compared to the double-susceptible population.

The plateau factor (PLAT as described previously) (8, 12, 13), represents the probability of successful replication. The first-order growth rate constant (k_{12}) was defined by the mean generation time (MGT) for each of the bacterial subpopulations (k_{12} = 60/MGT). The first-order replication rate constant (k_{21}) was set to 50 h^{-1} (7). The KC_{50} is the antibiotic concentration required to achieve 50% of the maximum killing rate constant (K_{max}). The C_{MEM} and C_{CIP} were the concentrations of meropenem and ciprofloxacin in broth, and $Hill_{MEM}$ is the Hill coefficient for meropenem. The term SYN (*i.e*. mechanistic synergy) is described in equation 4.

Synergy modeling. We incorporated subpopulation synergy (*i.e.* meropenem killing the bacteria resistant to ciprofloxacin and *vice versa*) and mechanistic synergy (*i.e.* ciprofloxacin enhancing the killing by meropenem against one or multiple bacterial populations) (8, 12, 13). The mechanistic synergy was incorporated in the model using the equation described below (parameters are explained in **Table 2** and **S1**):

$$SYN = 1 - \left(\frac{I_{max,syn} \cdot C_{CIP}}{C_{CIP} + IC_{50,syn}} \right) \quad (4)$$

Observation model. An additive residual error model on a $\log_{10}$ scale was used to fit the $\log_{10}$ viability counts. When there was less than 2 colonies per plate observed, a residual error model that was previously described was utilized (3). Viable counts were plotted as 0 when below the limit of counting and when model-predictions were less than 0 $\log_{10}$ CFU/mL.

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Chapter 7

Conclusions and Future Directions

Chapter 7: Conclusions and Future Directions

Antimicrobial resistance is a global health threat and is especially problematic with *P. aeruginosa* infections. Respiratory infections of patients with CF have often been found to harbour *P. aeruginosa* exhibiting hypermutator phenotype. Hypermutable *P. aeruginosa* have an enhanced potential to adapt to their environment due to their increased mutation rate. Hence, these hypermutable strains can develop resistance more easily, thus posing a greater threat to patients with CF. The presence of *P. aeruginosa* in the lungs of patients with CF is also associated with increased rates of mortality. Antibiotics used against hypermutators have been found to be insufficient at suppression of bacterial regrowth and emergence of resistance, due to the amplification of pre-existing RMS. Treatment regimens are often based on the PK/PD indices, however further attention is needed to better inform the choice of therapy to treat an infection. Furthermore, combinations of available antibiotics are promising, however substantial gaps exist regarding whether these combinations will produce synergistic antibacterial effects against hypermutators. In addressing **Hypotheses 1 – 4** and **Project Aims 1 – 4**, this thesis aimed to evaluate dosage regimens that are currently used in patients with CF and identify effective synergistic antibiotic combinations to suppress the emergence of resistance often observed with hypermutators. The findings from chapters contained herein provide valuable insights into how shorter durations of exposure at the same *f*AUC/MIC for ciprofloxacin and tobramycin suppressed resistance emergence, the antibacterial effects of antibiotics in monotherapy against hypermutable *P. aeruginosa*, the prevalence of hypermutators in patients with CF from Australia and evaluation of promising, synergistic antibiotic combinations to combat these hypermutators.

In Chapters 2 and 3, we showed for the first time the impact of the shape of the concentration-time profile on the emergence and amplification of resistance against ciprofloxacin and

tobramycin. We showed that at the same *f*AUC/MIC, longer durations of ciprofloxacin and tobramycin exposures in monotherapy resulted in extensive emergence of resistance, whilst short durations of exposure did not amplify resistant populations. The results suggested that emergence of resistance was due to pre-existing resistant mutants. The findings from these studies indicated the potential to substantially reduce the emergence of resistance with improved fluoroquinolone and aminoglycoside dosage regimens, involving high-concentration, short-course regimens, in combination with a second antibiotic. Chapters 3 also demonstrated that tobramycin monotherapy led to increased resistance emergence for hypermutable PAOΔ*mutS* compared to PAO1. This highlighted the dire need to improve treatments for these pathogens that frequent the lungs of patients with CF. MBM well described the bacterial time-course of the total and resistant populations with different exposure levels of tobramycin.

The study in Chapter 4 enabled for the first time a thorough comparison of the antibacterial effects of antibiotics with different mechanisms of action in monotherapy against non-hypermutable and hypermutable laboratory strains over 48 h. It was demonstrated that emergence of resistance to all clinically important antibiotics tested was more prominent for hypermutable than non-hypermutable *P. aeruginosa*. The results in Chapter 4 indicated that monotherapy employing clinically viable concentrations of common antibiotics was an ineffective means of combating hypermutable *P. aeruginosa* due to the subsequent less-susceptible populations that emerge. However, the synergistic combination of two-fast acting antibiotics, ciprofloxacin and tobramycin, demonstrated promise to combat these difficult hypermutators, with resistance suppression observed for both non-hypermutable PAO1 and hypermutable PAOΔ*mutS*. The tobramycin and ciprofloxacin combination warrants further evaluation in dynamic *in vitro* systems that simulate the pharmacokinetics as observed in patients.

In Chapter 5, a comprehensive characterisation was undertaken for 59 clinical isolates obtained from chronic respiratory infections of CF patients from an Australian clinic. It was found that 22% of isolates were hypermutators, which was comparable with other geographical locations. The susceptibility of these hypermutators was shown to be substantially reduced for all clinically relevant antibiotics in comparison to non-hypermutators, with a greater proportion of MDR strains among the hypermutators. Interestingly, whole genome sequencing of the clinical hypermutators revealed that these isolates were genetically diverse from each other, however had relatedness to two common epidemic strains, DK2 and AES-1R. This was the first comprehensive characterisation of hypermutators of a cohort of CF patients from a single Australian clinic, that determined the current prevalence of hypermutators in Australia.

The susceptibilities of the clinical hypermutator strains, and preliminary static concentration time-kill studies, suggested that the combination of ciprofloxacin and meropenem should be evaluated. In Chapter 6, this combination was found to be promising against clinical hypermutators. The 72-h SCTK studies showed that concentrations representative of clinically achieved ELF concentrations were synergistic against both hypermutable and non-hypermutable *P. aeruginosa*, including three clinical hypermutators. Subsequently, the combination was evaluated in the dynamic HFIM system over 8 days, simulating ELF concentration-time profiles as would be observed in CF patients. The combination demonstrated synergistic bacterial killing and resistance suppression against a clinical hypermutator. The highest clinically recommended dosage regimens, 400 mg ciprofloxacin administered thrice daily as a 1 h infusion and 2 g meropenem administered thrice daily as a 3 h infusion, were not sufficient in monotherapy against a clinical hypermutator. However, these regimens in combination led to extensive bacterial killing and the suppression of resistance emergence over 8 days. This is one of the first

dynamic studies evaluating an antibiotic combination against a clinical *P. aeruginosa* hypermutator. The time-courses of bacterial killing and regrowth were successfully described by an MBM including subpopulation and mechanistic synergy. Modelling suggested that the mechanistic synergy was due to ciprofloxacin enhancing the antibacterial effects of meropenem. This promising combination warrants further investigation.

Future studies including optimising the dosage regimens of tobramycin with ciprofloxacin and meropenem with ciprofloxacin in dynamic *in vitro* models are warranted. Both front-loaded ciprofloxacin regimens and further evaluation of continuous meropenem infusion should be examined. Subsequently, these optimised antibiotic combination regimens should be evaluated against MDR hypermutators. Investigation in a dynamic *in vitro* biofilm model is also warranted, as biofilm growth is relevant for the later stages of chronic lung infections. Patients with CF are often infected with multiple different pathogens, hence evaluating these antibiotic combination regimens against polybacterial infections should be considered in the future. The hollow fibre infection model represents the situation in immunocompromised patients, thus studies a worst-case scenario. However, an immune system effect could be incorporated through mathematical modelling including data from animal models. Studies in immunocompetent *in vivo* animal models could also be considered to support extrapolation to immunocompetent patients. These studies, combined with MBM and Monte Carlo simulations, would support the translation to future evaluations in patients.

Overall, we demonstrated the promise of current antibiotics in synergistic combinations to be effective against problematic hypermutable *P. aeruginosa* strains. The findings of the studies in this thesis provide implications for the use of traditional PK/PD targets, particularly against hypermutators, and guidance for the selection of synergistic antibiotic combinations to combat

these pathogens. It was concluded that high-concentration, short durations of antibiotic exposure (at the same *f*AUC/MIC) maximised bacterial killing and reduced resistance emergence in *P. aeruginosa* for both ciprofloxacin and tobramycin (Chapters 2 and 3). Exposure of hypermutable and non-hypermutable *P. aeruginosa* to commonly used antibiotics in monotherapy showed that resistance emergence was observably greater in the hypermutable laboratory strain compared to the non-hypermutable strain (Chapter 4). The combinations of tobramycin or meropenem in combination with ciprofloxacin showed synergy and suppression of resistance (Chapters 4 and 6). The characterisation of the *P. aeruginosa* isolates from respiratory infections of CF patients revealed hypermutators had a similar prevalence to other geographical locations around the world (Chapter 5). Thus the need to further investigate more promising antibiotic therapies to treat infections caused by these hypermutable strains was highlighted. Static *in vitro* experiments allowed us to assess the ciprofloxacin plus meropenem combination in three *P. aeruginosa* isolates (Chapter 6). Notably, we successfully utilised MBM to quantitatively describe the time-course of bacterial growth, killing and resistance emergence for hypermutable and non-hypermutable *P. aeruginosa* (Chapters 3 and 6). The dynamic *in vitro* model along with this MBM clarified that ciprofloxacin plus meropenem combination produced substantial bacterial killing and resistance suppression in a clinical *P. aeruginosa* hypermutator (Chapter 6). In this thesis, the PK/PD assessment and MBM approaches used have significantly contributed to identifying clinically relevant, synergistic antibiotic combinations to combat hypermutable *P. aeruginosa* in patients with CF, which are promising for further evaluation in the future.