MONASH University

Optimising use of fosfomycin against multidrug-resistant *Pseudomonas aeruginosa*

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Faculty of Pharmacy and Pharmaceutical Sciences

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This thesis is dedicated to my parents, Peter and Christine, who have done everything humanly possible to provide the best life for me

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Abstract

Rising resistance to most antibiotics coupled with a marked decline in antibiotic discovery has led to a recent increase in the use of fosfomycin for treatment of systemic infections due to multidrug-resistant (MDR) bacteria, including MDR *Pseudomonas aeruginosa*. Fosfomycin retains significant activity against a range of MDR Gram-positive and Gram-negative bacteria, including *P. aeruginosa*, and has an excellent safety profile. However, fosfomycin was identified and developed prior to the introduction of modern drug development and approval procedures. Consequently, substantial gaps in our knowledge of the pharmacokinetic (PK) and pharmacodynamic (PD) properties of fosfomycin currently exist. These properties are required to optimize the use of fosfomycin and improve patient care and outcomes by maximizing bacterial killing and minimizing the emergence of resistance.

The *in vitro* PD properties of fosfomycin, namely the minimum inhibitory concentrations (MICs), bacterial killing (including the effect of inoculum), population analysis profiles (PAPs) and the post-antibiotic effect (PAE) were systematically investigated against clinical isolates of *P. aeruginosa,* including MDR isolates. MICs ranged from 1 – >512 mg/L, with 61% of isolates considered fosfomycin-susceptible (MIC ≤64 mg/L). Baseline PAPs indicated heteroresistance in all isolates tested. Time-kill studies showed moderate, time-dependent killing at the low inoculum, with regrowth of fosfomycin-resistant colonies. Bacterial killing was virtually eliminated at the high inoculum. The data suggest fosfomycin monotherapy may be problematic for the treatment of infections caused by *P. aeruginosa*.

Resistance is known to develop rapidly when fosfomycin is used as monotherapy, particularly against *P. aeruginosa*, and combination regimens with a second antibiotic have

been suggested to overcome this potential problem. Bacterial killing and resistance emergence with fosfomycin monotherapy and in combination with tobramycin, polymyxin B or ciprofloxacin were systematically investigated using static-time kill methodology (inocula ~10^6 cfu/mL). The combination of fosfomycin with polymyxin B or tobramycin at clinically relevant concentrations substantially improved bacterial killing of fosfomycin-susceptible *P. aeruginosa*, while fosfomycin plus ciprofloxacin substantially improved bacterial killing of fosfomycin-resistant isolates. No combination was able to suppress the emergence of fosfomycin resistance.

In brief, this thesis established a range of PD properties for fosfomycin against *P. aeruginosa*. It shows for the first time, as far as we are aware, in *P. aeruginosa* the presence of fosfomycin heteroresistance and that the antimicrobial activity of fosfomycin is time-dependent. The first systematic investigation into fosfomycin combination therapy, as far as we are aware, is undertaken, including the first examination of the emergence of fosfomycin resistance with combination therapy over time. The studies reported here will increase our understanding of fosfomycin PD, both in mono- and combination-therapy, and will assist in the design of optimal fosfomycin dosing regimens that will maximize bacterial killing and minimize the emergence of fosfomycin resistance.

Thesis including published works General Declaration

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 papers published in, or submitted to, peer reviewed journals. The core theme of the thesis is the pharmacodynamics of fosfomycin against *Pseudomonas aeruginosa*. 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 under the supervision of Dr Phillip Bergen (primary supervisor), Prof Carl Kirkpatrick, A/Prof Michelle McIntosh and Prof Anton Peleg. The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of Chapters 2 and 3 my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status	Nature and extent (%) of students contribution
2	*In vitro* pharmacodynamics of fosfomycin against clinical isolates of *Pseudomonas aeruginosa.*	Published	65%
3	Clinically relevant concentrations of fosfomycin combined with polymyxin B, tobramycin, or ciprofloxacin enhance bacterial killing of *Pseudomonas aeruginosa* but do not suppress the emergence of fosfomycin resistance.	Submitted	80%

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: [redacted] Date: **17/12/2015**

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student and co-authors' contributions to this work.

Main Supervisor signature: [redacted] **Date:** 17/12/2015

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Publications

This thesis is a compilation of the following manuscripts:

1. **Walsh CC,** McIntosh MP, Peleg AY, Kirkpatrick CM, Bergen PJ. *In vitro* pharmacodynamics of fosfomycin against clinical isolates of *Pseudomonas aeruginosa*. *Journal of Antimicrobial Chemotherapy* **70**(11): 3042-3050.

2. **Walsh CC,** Landersdorfer CB, McIntosh MP, Peleg AY, Hirsch EB, Kirkpatrick CM, Bergen PJ. Clinically relevant concentrations of fosfomycin combined with polymyxin B, tobramycin, or ciprofloxacin enhance bacterial killing of *Pseudomonas aeruginosa* but do not suppress the emergence of fosfomycin resistance. (Manuscript accepted).

Presentations

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

1. **Walsh, CC,** AY Peleg, CM Kirkpatrick, MP McIntosh, and PJ Bergen. MIC distributions of fosfomycin for Australian Isolates of *Pseudomonas aeruginosa*. Monash University Pharmacy and Pharmaceutical Sciences 9thAnnual Postgraduate Symposium, Melbourne, Australia (poster presentation).

2. **Walsh, CC,** AY Peleg, CM Kirkpatrick, MP McIntosh, and PJ Bergen. *In vitro* pharmacodynamics of fosfomycin against clinical isolates of *Pseudomonas aeruginosa.* Monash University Pharmacy and Pharmaceutical Sciences 10thAnnual Postgraduate Symposium, Melbourne, Australia (oral presentation). This presentation was judged the winner for most outstanding oral presentation.

3. **Walsh, CC,** AY Peleg, CM Kirkpatrick, MP McIntosh, and PJ Bergen. Synergistic Killing of *Pseudomonas aeruginosa* (Pa) by Fosfomycin (Fos) plus Polymyxin B (PMB) in a Static Time-Kill Model. Abstract A-946. The 55th Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC)/The 29th International Congress on Chemotherapy and Infection (ICC) 2015, San Diego, CA, USA (poster presentation).

Glossary of abbreviations

Asp	aspartic acid
ATCC	American Type Culture Collection
AUC	area under the concentration-time curve
cAMP-CRP	cyclic adenosine monophosphate receptor protein complex
cfu or CFU	colony forming units
CLSI	Clinical and Laboratory Standards Institute
C_{max}	maximum serum concentration
C_{min}	trough serum concentration
CSF	cerebrospinal fluid
CVVH	continuous venovenous hemofiltration
Cys	cysteine
ESBL	extended-spectrum beta-lactamase
ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	United States Food and Drug Administration

FEV_1	forced expiratory volume in 1 second
FICI	fractional inhibitory concentration index
G3P	glycerol-3-phosphate
G6P	glucose-6-phosphate
GlpT	ι-α-glycerophosphate (glycerol-3-phosphate [G3P]) system
h	hour/hours
HPLC	high-performance liquid chromatography
IDSA	Infectious Diseases Society of America
i.m.	intramuscular/intramuscularly
i.v.	intravenous/intravenously
L	litre
MDR	multidrug-resistant
MIC	minimum inhibitory concentration
min	minute
mL	millilitre
MRSA	methicillin-resistant *Staphylococcus aureus*
MurA	uridine diphosphate (UDP)-*N*-acetylglucosamine enolpyruvyl transferase

PAE	post-antibiotic effect
PD	pharmacodynamics
PEP	phosphoenolpyruvate
PK	pharmacokinetic
PK/PD	pharmacokinetic/pharmacodynamic
$t_{1/2}$	half-life
$\%T_{>MIC}$	cumulative percentage of a 24-h period that the drug concentration exceeds the MIC at steady state
UDP	uridine diphosphate
UhpT	hexose-phosphate (glucose-6-phosphate [G6P]) uptake system
UTI	urinary tract infection
V_d	volume of distribution
WHO	World Health Organization
XDR	extensively drug-resistant

Chapter One

General Introduction

Project outline

1.1 Statement of the problem

Bacterial infections are a major cause of morbidity and mortality across the globe and the third-leading cause of death in economically advanced countries.[1-4] Antimicrobial resistant bacterial infections are particularly problematic. According to the World Health Organisation (WHO), antimicrobial resistance is one of the three greatest threats to human health.[5] A group of pathogens known as the ESKAPE pathogens – *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species – are responsible for the majority of nosocomial bacterial infections and are known to 'escape' the bactericidal effects of many antibiotics through a wide range of drug resistance mechanisms.[6] These ESKAPE pathogens have outpaced the drug discovery process (Figure 1.1).[7] A recent WHO report on antimicrobial resistance noted that the problem is so serious that a post-antibiotic era – in which common infections and minor injuries could prove fatal – is a very real possibility for the 21st century.[1] Indeed, this possibility is beginning to be realized with the emergence of pandrug-resistant bacteria – that is, bacteria resistant to all currently available antibiotics. For Gram-negative bacteria pandrug-resistant isolates of *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae* have already been observed.[8]

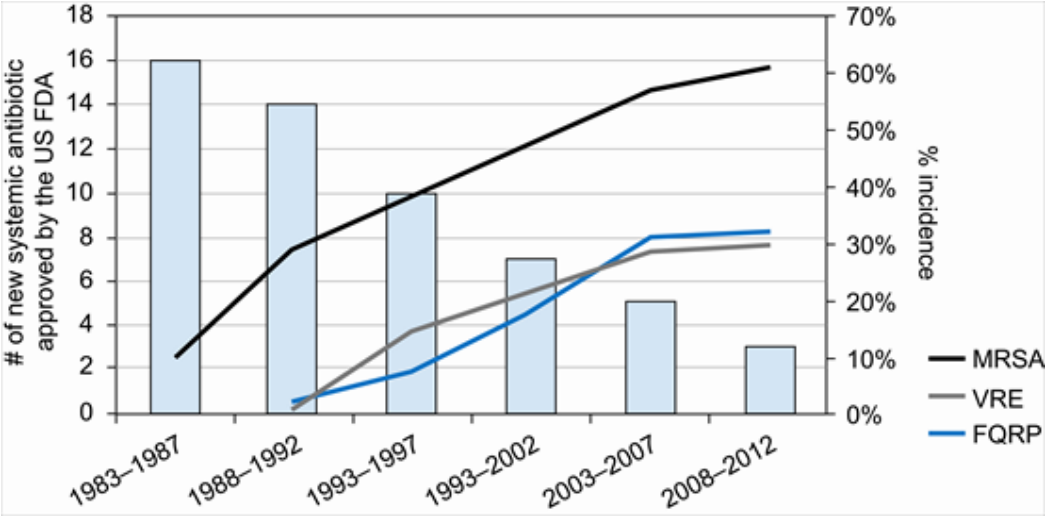

Figure 1.1. Declining antibiotic development, superimposed by the increase in the prevalence of methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE) and fluoroquinolone-resistant *P. aeruginosa* (FQRP). Figure reproduced from Allen and Deschambeault, [9] with permission.

The Gram-negative bacterium *P. aeruginosa* is a particularly problematic pathogen. A member of the ESKAPE pathogens,[6] *P. aeruginosa* has been identified by the Infectious Diseases Society of America (IDSA) as one of the top six pathogens threatening healthcare systems.[10] This opportunistic pathogen has been implicated in many types of infections including those of the upper and lower airways (including ventilator-associated pneumonia), bloodstream, urinary tract, bone, joint, skin, soft tissue and ophthalmic infections.[11-14] Infections caused by *P. aeruginosa* are especially troublesome in patients with underlying conditions such as cystic fibrosis, the immunocompromised and the critically ill,[15-17] and are responsible for increased lengths of hospital stay, need for surgical intervention, severe illness, death, and increased cost.[18-21]

In light of the emerging threat of multidrug-resistant (MDR) bacteria discussed above there is an urgent need for new antibiotics with activity against these organisms, particularly *P. aeruginosa*.[5,22,23] While a small number of new antibiotics active against Gram-positive organisms have been approved over the last decade,[24] the situation is quite different for MDR Gram-negative bacteria.[7] Between 2000 and 2010 only ten new systemic antibiotics, four with novel mechanism of actions, were approved for treatment of infections caused by Gram-negative bacilli; two of these new antibiotics (one with a novel mechanism) have been withdrawn due to adverse effects.[22] As of 2013 seven intravenous (i.v.) antibiotics were currently undergoing Phase 2 and 3 clinical trials.[22] However, these agents have either no, or very limited, activity against MDR *P. aeruginosa* (Table 1.1). Thus there remains a critical need for antibiotics which can effectively treat this organism. Unfortunately the development process for new antibiotics takes many years.[25] In the interim, one option currently being utilised is the re-introduction of older, rarely used antibiotics such as fosfomycin and the polymyxins (colistin and polymyxin B).[26-31] These antibiotics still retain significant activity against many MDR bacteria, including *P. aeruginosa*.[26,31-33]

Table 1.1. Intravenous antimicrobials active against Gram-negative bacilli in advanced (Phase 2 or 3) clinical development. Table reproduced from Boucher *et al.*, [22] with permission.

Product	Class (Mechanism of Action)	Novel Mechanism of Action?	Status	Activity Targets							
				Enterobacteriaceae			*Pseudomonas aeruginosa*			*Acinetobacter* species	
				ESBL	sCBP	mCBP	WT	MDR	mCBP	WT	MDR
Ceftolozane/tazobactam (CXA-201; CXA-101/tazobactam)	Antipseudomonal cephalosporin/BLI combination (cell wall synthesis inhibitor)	No	Phase 3 (cUTI, cIAI)	Yes	No	No	Yes	IE	No	No	No
Ceftazidime-avibactam (ceftazidime/NXL104)	Antipseudomonal cephalosporin/BLI combination (cell wall synthesis inhibitor)	No	Phase 3 (cIAI)	Yes	Yes	No	Yes	IE	No	No	No
Ceftaroline-avibactam (CPT-avibactam; ceftaroline/NXL104)	Anti-MRSA cephalosporin/ BLI combination (cell wall synthesis inhibitor)	No	Phase 2 (cUTI, cIAI)	Yes	Yes	No	No	No	No	No	No
Imipenem/MK-7655	Carbapenem/BLI combination (cell wall synthesis inhibitor)	No	Phase 2 (cUTI, cIAI)	Yes	Yes	No	Yes	IE	No	IE	No
Plazomicin (ACHN-490)	Aminoglycoside (protein synthesis inhibitor)	No	Phase 2 (cUTI)	Yes[a]	Yes[a]	IE	No	No	No	No	No
Eravacycline (TP-434)	Fluorocycline (protein synthesis inhibitor targeting the ribosome)	No	Phase 2 (cIAI)	Yes[a]	Yes	IE	No	No	No	IE	IE
Brilacidin (PMX-30063)	Peptide defence protein mimetic (cell membrane disruption)	Yes?	Phase 2 (ABSSSI)	Yes	IE	IE	IE	IE	IE	No	No

Activity based on available information.

Abbreviations: ABSSSI, acute bacterial skin and skin structure infection; BLI, β-lactamase inhibitor; cIAI, complicated intra-abdominal infection; cUTI, complicated urinary tract infection; ESBL, extended-spectrum β-lactamase producers; IE, insufficient evidence available; mCPB, metallo-carbapenamase producers (e.g., NDM, VIM, IMP); MDR, multidrug-resistant pattern including co-resistances to aminoglycosides (amikacin, gentamicin, tobramycin), fluoroquinolones, tetracyclines, and broad-spectrum β-lactams by various mechanisms carried on common genetic elements; MRSA, methicillin-resistant *Staphylococcus aureus*; sCBP, serine carbapenemase producers such as KPC; WT, wild-type pattern for species.

[a] Incomplete coverage of some species (*Proteus mirabilis* and indole-positive *Proteus* species).

Antibiotics such as fosfomycin and the polymyxins were developed decades ago[34-37] and thus were not subjected to drug development procedures now mandated by international drug regulatory authorities.[38] As a consequence there has been a lack of critical information on the pharmacokinetics (PK) and pharmacodynamics (PD) of these agents to inform optimal dosing strategies, that is, strategies which maximise bacterial killing and minimize both the emergence of resistant bacteria and adverse effects.[30,39] Over the last 10 to 15 years, however, significant research has been undertaken on the polymyxins which has recently resulted in publication of the first scientifically-based dosage guidelines for colistin in a variety of patient groups.[40] The 'colistin story' has become the prototype for the redevelopment process required for these older agents.[30,39] Unfortunately while the polymyxins (primarily colistin) have assumed the role of 'salvage' therapy in patients with otherwise untreatable infections caused by MDR Gram-negative bacteria, including *P. aeruginosa*, they cause dose-limiting nephrotoxicity in up to 50% of patients.[40-42] Additionally, polymyxin resistance is increasingly being reported.[43-47] Thus while the polymyxins have temporarily provided another weapon in the clinician's arsenal of antibiotics for treatment of infections caused by *P. aeruginosa* more, and safer, alternatives are still urgently required.

A similar redevelopment process to that undertaken for the polymyxins is required for the remaining older agents where PK, PD, and other scientific information essential in determining optimal dosage regimens is yet to be determined.[38] Fosfomycin retains significant activity against a range of MDR Gram-positive and Gram-negative bacteria[48-59] and, in stark contrast to the polymyxins, has considerably less toxicity.[26,60] Given this activity, favourable adverse effect profile and increasing resistance to other agents there has recently been an increase in the use of fosfomycin for the treatment of systemic infections caused by MDR organisms.[32,50-52,61-63] Several preclinical and clinical studies have shown fosfomycin to be a

promising agent, especially as part of combination therapy, for the treatment of various infections caused by both MDR Gram-negative and Gram-positive organisms, including *P. aeruginosa*.[50-52,56,61] However, unlike the polymyxins fosfomycin has not been subjected to an intensive program of research aimed at elucidating critical PD and PK information necessary for determining optimal therapy against any organism. This thesis will focus on the use of fosfomycin, alone and as part of combination therapy, for the treatment of systemic infections caused by *P. aeruginosa* with the aim of increasing our understanding of essential PD information required for optimisation of its clinical use against this pathogen.

1.2 Fosfomycin

1.2.1 History of fosfomycin

The antibiotic fosfomycin, also termed phosphomycin and originally named phosphonomycin, was first isolated in Spain in 1969 as a product of *Streptomyces* species.[34] A natural antibiotic compound produced by several species of *Streptomyces* and *Pseudomonas*, it is now produced synthetically for clinical use.[25] During initial pharmacological and clinical testing in the United States low doses, mostly of the oral calcium salt formulation which has relatively poor bioavailability (discussed in Section 1.5), were employed resulting in poor clinical efficacy for a variety of infections.[64,65] Consequently, fosfomycin was not seen as a particularly useful antibiotic and testing was abandoned.[64] However, at the same time research with larger parenteral doses was being conducted in Spain which provided more promising results.[65] Despite this, fosfomycin has until recently been restricted primarily to use of the oral formulation in the treatment of urinary tract infections (UTIs), with the parenteral formulation available in only a few countries.[32] However, in the last 5 to 10 years there has been renewed interest in i.v. fosfomycin for the treatment of non-UTIs.[32,50-52,61-63] This interest has been driven by a need for alternative treatments to help combat the ever-growing numbers of MDR

bacteria as discussed above (Section 1.1), combined with the limited number of new antibiotics which are likely to become available in the foreseeable future.[22]

Given the scenario outlined above, considerable attention is now being directed towards redeveloping this older 'forgotten' antibiotic.[55,66-68] Until the recent reintroduction of polymyxins for the treatment of infections caused by MDR Gram-negative bacteria there had been little historical precedent for the reintroduction of older, infrequently used antibiotics such as fosfomycin into general clinical use. The process undertaken for the successful reintroduction of colistin can serve as a prototype for resurrection of other 'forgotten' antibiotics such as fosfomycin. Similar investigations to those which led to the rational dosing of colistin are required for fosfomycin. With the emergence of resistance to fosfomycin known to develop rapidly *in vitro* and concerns that this may translate to high resistance rates clinically,[69,70] there is an urgent need to better understand the PK and PD properties of fosfomycin in order to optimize its use. If this goal can be realized fosfomycin may serve as a useful treatment option for MDR infections, at least until newer agents become available.[50,51,68,71]

1.2.2 Chemistry and commercial formulations

Fosfomycin represents its own antibiotic class[25] and is chemically unrelated to any other antibiotic.[55] A phosphonic acid derivative[55] with the chemical name (-)-cis-1,2-epoxypropyl-phosphonic acid[34] and empirical formula $C_3H_7O_4P$, it has the smallest molecular weight (138 g/mol) of all antibiotics.[25,33 55] It comprises a phosphate group, propyl group and epoxide ring (Figure 1.2).[72]

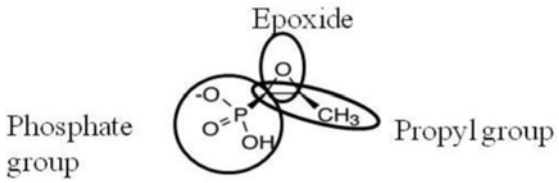

Figure 1.2. Chemical structure of fosfomycin. Figure reproduced from Castañeda-García *et al.*,[72] with permission.

Fosfomycin is available in three salt forms – fosfomycin tromethamine (or trometamol; molecular formula, $C_3H_7O_4P \cdot C_4H_{11}NO_3$; molecular weight, 259.2 g/mol), fosfomycin calcium (molecular formula, $C_3H_5CaO_4P$; molecular weight, 176.1 g/mol), and fosfomycin disodium (molecular formula, $C_3H_5Na_2O_4P$; molecular weight, 182.0 g/mol).[55] Fosfomycin calcium is a relatively insoluble salt taken orally,[73] fosfomycin tromethamine is a soluble salt taken orally,[74] and fosfomycin disodium a highly soluble salt given i.v. and occasionally intramuscularly (i.m.).[73] Fosfomycin tromethamine comes as a sachet of powder that is mixed with water before ingestion.[75] The inactive salt component (tromethamine) of fosfomycin tromethamine contributes almost one half of the dose reported (i.e. 1 g of fosfomycin tromethamine = 0.53 g of fosfomycin and 0.47 g of tromethamine; each sachet contains 5.631 g of fosfomycin tromethamine, equivalent to 3 g of fosfomycin).[76] Similarly, i.v. fosfomycin (as the sodium salt) contains only 75.9% fosfomycin; every 1 g of fosfomycin i.v. contains 0.33 g of sodium. This is important as a high sodium intake may be a limitation in patients with renal or heart failure (see Section 1.3.1.2).[77,78] Combination products for inhalation have recently been developed containing fosfomycin disodium and tobramycin sulphate, as well as fosfomycin disodium and amikacin base.[79,80]

1.2.3 Dosage regimens

For the tromethamine salt, the United States Food and Drug Administration (FDA) approved regimen for uncomplicated UTIs is a single 3 g oral dose.[75] For resistant organisms or in complicated UTIs more frequent doses over longer durations (typically 3 g every 2 – 3 days for ~1 – 3 weeks) are administered.[26,55,66] Given its lower bioavailability compared to the tromethamine salt, fosfomycin calcium requires more frequent dosing (typically 0.5 – 1 g every 6 – 8 h)[81] and has therefore fallen out of favor. As a consequence, most clinical trials of oral therapy use the tromethamine salt.[26,55,66,82-84] Although studies utilizing fosfomycin calcium for treatment of systemic infections were undertaken in the 1970s,[73,85,86] since then the i.v. formulation (fosfomycin disodium) has been used exclusively to treat infections other than UTIs.[32]

Very little clinical data is currently available to support the use of fosfomycin for non-UTI infections. For fosfomycin disodium, typical daily doses in patients with normal renal function range from 12 to 16 g administered 6-, 8- or 12-hourly i.v. as a bolus or 30 – 60 minute infusion.[33,55,87] However, daily doses as low as 1 g and as high as 24 g have been reported,[33,88] as have longer infusion times (of 4 h[89]). Variability in the dose administered is most likely due to the lack of information regarding appropriate PK/PD targets for maximal bacterial effect. It has been recommended that the dose of i.v. fosfomycin be reduced in renal impairment when creatinine clearance is < 50mL/min,[55,90] although there appears to be little to no guidance in the literature regarding an appropriate dose reduction. Fosfomycin is removed through hemodialysis and as such it is recommended the usual dose be administered after hemodialysis.[91-93] In anuric intensive care patients receiving continuous venovenous hemofiltration (CVVH), doses similar to that used in normal renal function (8 g 12-hourly) have been recommended.[94] Due to the PK changes that occur in critically ill patients (such as

enhanced or reduced renal clearance, and altered volume of distribution [V_d]), dosing requirements in these patients are likely to be different to those needed for non-critically ill patients.[71] However, more PK and PD information on fosfomycin generally, and in this patient group in particular, is required before any dosing recommendations for critically ill patients can be made.

1.2.4 Antimicrobial spectrum

Fosfomycin is a broad spectrum bactericidal antibiotic active against a range of Gram-positive and Gram-negative bacteria, including many MDR species. *In vitro* studies indicate fosfomycin remains highly active against many of the most problematic pathogens such as *P. aeruginosa* (including a minor but important subset of MDR *P. aeruginosa*),[48,49,95-97] methicillin-resistant *Staphylococcus aureus* (MRSA),[50,98] and many *Enterobacteriaceae* with MDR and extensively drug-resistant (XDR) phenotypes including extended-spectrum beta-lactamase (ESBL) producing *E. coli* and carbapenem-resistant *K. pneumoniae*.[31,48,52-59,96,99-102] Fosfomycin is also active against *Serratia marcescens*.[31] Some bacterial species are inherently resistant to fosfomycin including *A. baumannii*,[31,48,96,103] *Bacteroides fragilis*,[104] and *Listeria monocytogenes*.[32]

The current Clinical and Laboratory Standards Institute (CLSI)[105] and European Committee on Antimicrobial Susceptibility Testing (EUCAST)[106] minimum inhibitory concentration (MIC) interpretative standards for fosfomycin using the agar dilution method are shown in Table 1.2. Fosfomycin has been used primarily to treat UTIs, where reports of resistance to fosfomycin are rare despite many years of use.[32,107-109] A recent review reported that fosfomycin susceptibility of Gram-negative urinary isolates has remained relatively stable over time.[110] However, the reported rates of resistance in *P. aeruginosa* for systemic infections are quite variable and may be due, in part, to a lack of universally accepted fosfomycin

breakpoints.[51] It has also been suggested that anti-pseudomonal activity may be population dependent, and thus familiarity with local susceptibility patterns would be required for optimal use.[111] In this regard we report for the first time in Chapter 2 of this thesis the susceptibility of *P. aeruginosa* isolates to fosfomycin within an Australian context. The current low rates of resistance to fosfomycin reported in many bacterial species may be due to its limited use in many countries[25] and its absence from bioindustry and animal husbandry.[112]

Table 1.2. Current minimum inhibitory concentration (MIC) interpretive standards for fosfomycin using agar dilution.

Organism [a]		MIC Interpretive Standard (mg/L)		
		S	I	R
CLSI				
	Enterobacteriaceae	≤64	128	≥256
	Enterococcus species	≤64	128	≥256
EUCAST				
	Enterobacteriaceae	≤32	-	>32
	Staphylococcus species	≤32	-	>32

S, Susceptible; I, Intermediate; R, Resistant; CLSI, Clinical and Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing. Values shown are for i.v. fosfomycin.

[a] Neither CLSI nor EUCAST currently provide breakpoints for *P. aeruginosa*.

1.2.5 Clinical uses and adverse effects

1.2.5.1 Clinical uses:

Fosfomycin is most commonly used orally (as fosfomycin tromethamine) for UTIs caused by a variety of pathogens including *P. aeruginosa*, *E. coli*, *Klebsiella*, *Serratia*, and *Enterobacter* species, where high concentrations in the urine (typically ~1000 – 4000 mg/L) are achieved and persist for prolonged periods.[32,55,56,107,113-115]

In Australia, fosfomycin in any form is only available under the Therapeutic Goods Administration Special Access Scheme[116] and as such is not widely used. In the United States, fosfomycin is approved for oral use only for treatment of uncomplicated cystitis.[117] In Germany, France, Spain, and Japan it is available for i.v. use in systemic infections.[26] Other than UTIs, fosfomycin has been used to treat gastrointestinal infections,[118-120] pneumonia,[61,89,121] osteomyelitis,[122-124] meningitis,[125,126] diabetic foot infections,[127-129] typhoid fever,[130,131] prophylaxis of post-surgical infections,[132,133] obstetric and gynecological infections,[134] and sepsis.[135]

Although use of fosfomycin for the treatment of non-UTIs is still relatively uncommon, there has recently been renewed interest in the use of i.v. fosfomycin for the treatment of systemic infections caused by Gram-positive and Gram-negative MDR bacteria, including respiratory exacerbations involving *P. aeruginosa* in patients with cystic fibrosis.[32,50-52,62,63] A recent study concluded fosfomycin had excellent clinical success in treating critically ill patients infected with MDR and pandrug-resistant *P. aeruginosa*.[63] However, for infections other than those of the urinary and gastrointestinal tract the existing level of evidence for clinical effectiveness is not strong.[32] Unfortunately, given the lack of financial interest in this off-patent antibiotic there is little likelihood of comparative clinical trials based on current standards of antimicrobial drug development being conducted for the re-evaluation of fosfomycin. Rather, useful information on

the clinical applications of this agent is expected to derive from the assessment of the cumulative experience of its use as a last-resort therapeutic option, in conjunction with preclinical data.

The major consideration against the use of fosfomycin for systemic infections has been the risk of resistance emerging during therapy, related to a high mutation frequency observed *in vitro*.[69,70,110,136,137] Importantly for *P. aeruginosa*, the acquisition of fosfomycin resistance appears to come with no apparent fitness cost to the organism.[70] Thus, to date i.v. fosfomycin has mostly been used in combination with other antibiotics for systemic infections as it has been suggested combination therapy may protect against the emergence of resistance, although existing data are extremely limited.[70,110,138] Evidence for combination therapy reducing the emergence of resistance to fosfomycin is discussed in Section 1.7.

1.2.5.2 Adverse effects:

Fosfomycin is remarkably well tolerated and the reported adverse effects are usually mild, tolerable, and transient.[32,55,60,66,78,139] Adverse effects with oral use are most commonly gastrointestinal irritation, rash, vaginitis, headache, dizziness, fatigue and mild backache.[84,140] In a Japanese study following 35,481 patients for 6 years, only two cases of pseudomembranous colitis were observed after oral administration of fosfomycin calcium.[141] With i.v. administration, the most common adverse effect is hypokalemia which was reported in 5% of patients in a recent review examining 15 trials of parenteral fosfomycin in 578 patients,[60] although other studies have reported a higher incidence (~20 – 25%).[68,78] The hypokalemia associated with i.v. fosfomycin is thought to be due to an increase in urinary excretion of potassium in the distal part of the renal tubules.[78] Interestingly, in a study conducted by Florent *et al.*[78] the infusion time for fosfomycin appeared to influence the occurrence of hypokalemia. In that study fosfomycin infusion times (administered at a dose of 4 g 8-hourly in 86% of cases) were changed from 30 – 60 min to 4 h part-way through the study. With a 30 – 60 min infusion 19 of 57 (33%) patients experienced

hypokalemia, whereas none of 15 patients who were administered the antibiotic using the longer infusion experienced hypokalemia. The authors hypothesized that short infusions might explain the high rate of hypokalemia observed, but noted further investigation was required. On the other hand, an important safety consideration for parenteral fosfomycin is it high sodium content (1 g of i.v. fosfomycin possesses 330 mg of sodium) which may result in sodium overload and heart failure.[78,142] In comparison, piperacillin/tazobactam, considered to have a high sodium content, contains ~54 mg of sodium per gram of piperacillin.[143] Florent *et. al.*[78] reported cardiovascular adverse effect (hypertension or heart failure) due to sodium overload in 6% of patients. The high sodium content of parenteral fosfomycin should be taken into consideration when treating patients with underlying heart disease.

Other reported adverse effects of i.v. fosfomycin include injection site pain, peripheral phlebitis, rash, gastrointestinal disorders, and an increase in alanine aminotransferase.[32,55,60,66,78,139] The rates of hypersensitivity reactions are low, with only four cases of anaphylactic reactions associated with fosfomycin reported.[60] Interestingly, fosfomycin has been shown in preclinical studies to have a protective effect against nephrotoxicity when co-administered with nephrotoxic agents (aminoglycosides and vancomycin).[144-146] Fosfomycin has been found to inhibit gentamicin-induced lipid peroxidation by preventing the release of iron from renal cortex mitochondria, a known cause of renal toxicity due to the aminoglycoside antibiotic gentamicin.[144] Another suggested mechanism of fosfomycin-associated renal protection is changes in renal handling, most likely in tubular secretion/reabsorption of vancomycin.[145,147] Some clinical studies have also observed a lower than normal rate of nephrotoxicity in patients co-administered fosfomycin with nephrotoxic agents (e.g. gentamicin).[148] Such observations merit further investigation.

1.2.6 Mechanisms of antibacterial action and resistance

Fosfomycin inhibits an early step in bacterial cell wall synthesis.[55,72,149] Therefore, an understanding of the mechanism of action of fosfomycin requires knowledge of the structure of the bacterial cell envelope in general, and the bacterial cell wall in particular.

Structure of the bacterial cell envelope: The bacterial cell envelope (Figure 1.3) is comprised of multiple layers which surround and protect the cytoplasm, the layers differing between Gram-negative and Gram-positive bacteria.[150] Beginning from the structure closest to the cytoplasm and moving outwards, Gram-negative bacteria have a cell membrane followed by a periplasmic space, a thin peptidoglycan cell wall, and an outer membrane with lipopolysaccharide; Gram-positive bacteria lack the outer membrane and consist only of an inner cell membrane and a thicker layer of peptidoglycan.[150]

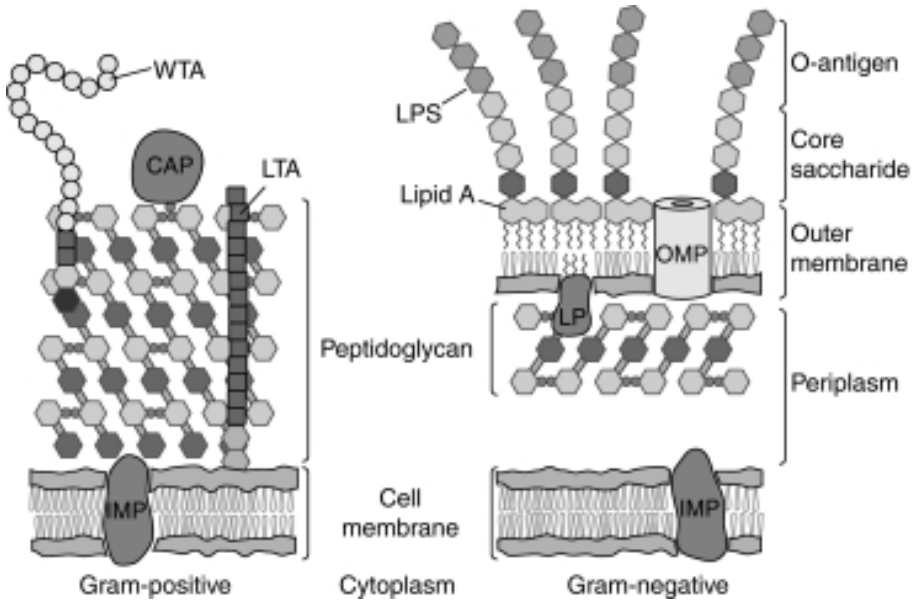

Figure 1.3. Depiction of Gram-positive and Gram-negative cell envelopes: CAP, covalently attached protein; IMP, integral membrane protein; LP, lipoprotein; LPS, lipopolysaccharide; LTA, lipoteichoic acid; OMP, outer membrane protein; WTA, wall teichoic acid. Figure adapted from Silhavy *et al.*,[150] with permission.

Structure of the bacterial cell wall: The bacterial cell wall is essential for bacterial survival. It protects the cell from osmotic pressure which would otherwise lyse and therefore destroy the cell.[151] It also protects from attack by other bacteria, viruses and protozoa, as well as toxic chemicals and immune responses such as from the human immune system.[151] In addition to this protective function, the cell wall provides a scaffold for other cellular elements to be inserted. The cell wall also maintains the shape of bacteria, which is important for survival.[151]

The bacterial cell wall is composed of peptidoglycan – a macromolecule chain comprised of two alternating sugars, *N*-acetylglucosamine (NAG or GlcNAc) and *N*-acetylmuramic acid (NAM or MurNAc), with an oligosaccharide side chain comprising five amino acids extending from NAM; these units (muropeptides) are covalently linked via the peptide side chain.[152] In Gram-negative bacteria the cell wall usually only consists of 1 – 2 peptidoglycan layers, whilst for Gram-positive bacteria it can contain around 20 – 50 layers.[152] The peptidoglycan layers are rigid, yet flexible and porous – these properties allow the cell wall to perform its various functions. The early steps in peptidoglycan synthesis, where the repeating muropeptide units are formed, occurs in the cytoplasm. From here, the muropeptides exit the cytoplasm by crossing the cytoplasmic membrane where they are subsequently polymerized and incorporated into the existing cell wall via penicillin binding proteins (Figure 1.4).[152]

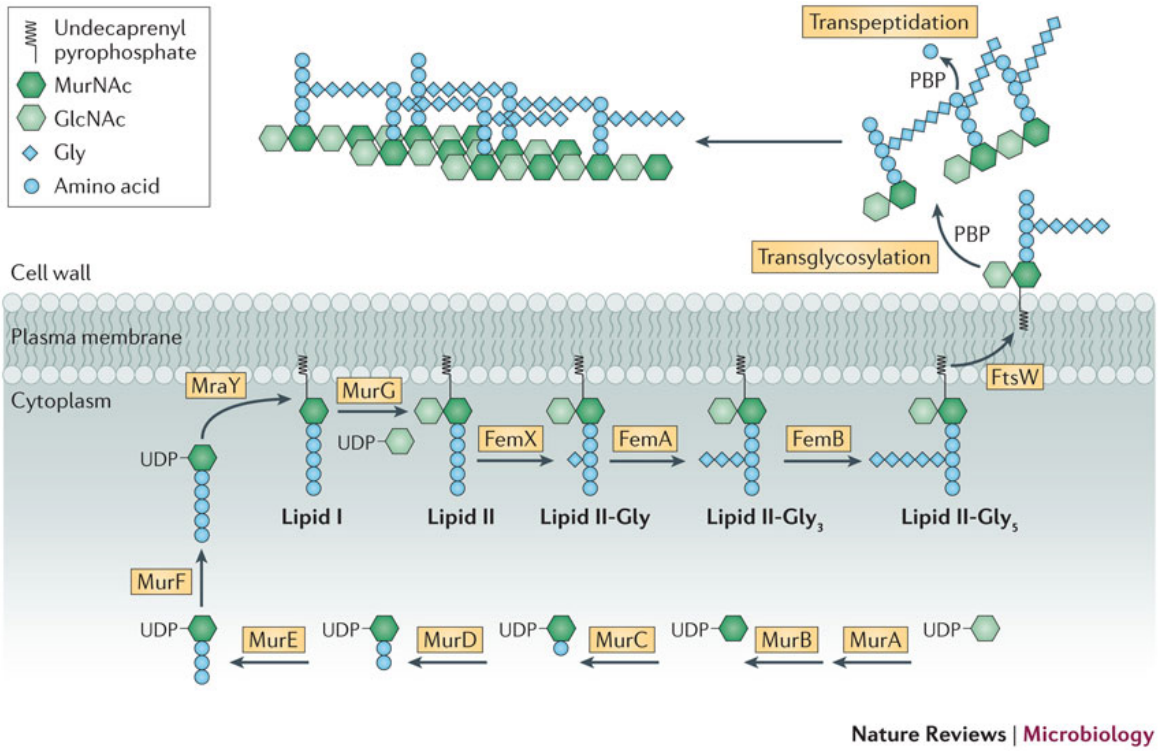

Figure 1.4. Peptidoglycan synthesis: Gly, glycan; PBP, penicillin-binding protein. Figure reproduced from Pinho *et al.*,[153] with permission.

Mechanism of action: To reach the site of action in the cytoplasm (discussed below), fosfomycin enters susceptible bacterial cells via either of two transport uptake systems: the ι- α- glycerophosphate (glycerol-3-phosphate [G3P]) system (GlpT) or the hexose-phosphate (glucose-6-phosphate [G6P]) uptake system (UhpT) (Figure 1.5).[154,155] Both GlpT and UhpT have low substrate specificity; GlpT not only transports G3P, and UhpT G6P, but they also transport fosfomycin and other chemicals such as arsenate and inorganic phosphate.[156,157] GlpT transporters exist in numerous bacteria and display a high degree of sequence conservation.[158] As will be discussed below, mutations in these transporters may confer fosfomycin resistance by reducing fosfomycin uptake into bacterial cells. Expression of GlpT and UhpT is induced by binding of their substrates, G3P and G6P, to the respective genes in the presence of cyclic adenosine monophosphate receptor protein complex (cAMP-CRP).[72] Both GlpT and UhpT are present in

E. coli and numerous *Enterobacteriaecea*,[149] whereas only GlpT is present in *P. aeruginosa*.[158] As a result, *glpT* is the only target gene whose inactivation confers antibiotic resistance in *P. aeruginosa*. [158]

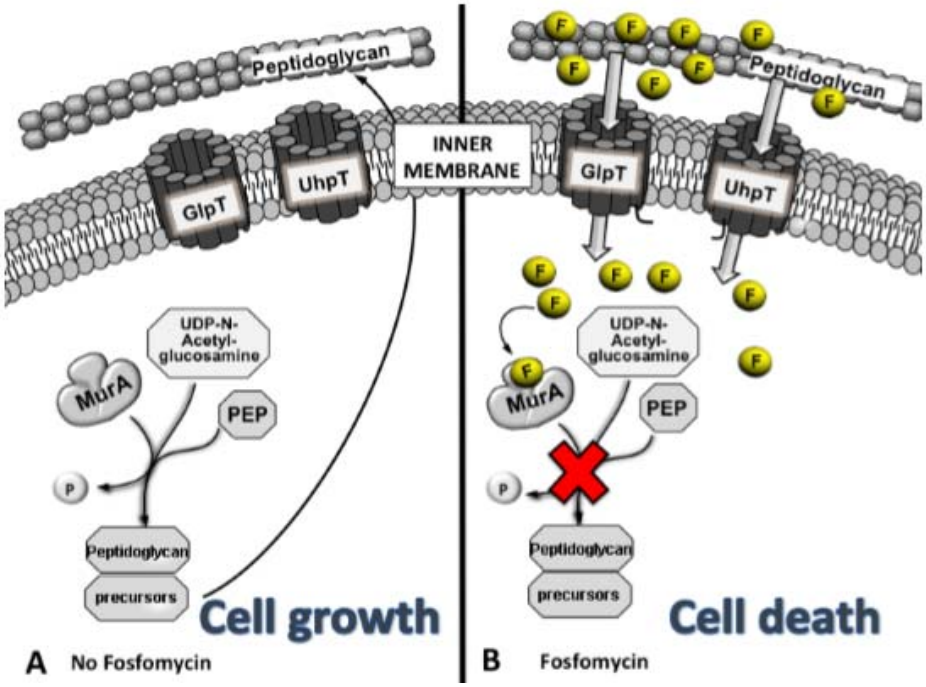

Figure 1.5. Although transporters are usually very selective, the chemical structure of fosfomycin mimics both G3P and G6P, which are transported under normal conditions. MurA catalyzes the formation of UDP-GlcNac-3-O-enolpyruvate, a peptidoglycan precursor, from UDP-GlcNAc and PEP during the first step of peptidoglycan biosynthesis, allowing cell growth (A). In contrast, when fosfomycin is present, it is transported inside the cell by GlpT and UhpT, blocking the UDP-GlcNac-3-O-enolpyruvate synthesis by mimicking the original substrate of MurA, PEP, avoiding cell wall synthesis and leading to cell death (B). For simplicity, only peptidoglycan and the inner membrane are shown. Figure reproduced from Castañeda-García *et al.*,[72] with permission.

Fosfomycin inhibits an early step in bacterial cell wall peptidoglycan synthesis (Figure 1.5).[55,72,149] Under normal circumstances the enzyme uridine diphosphate (UDP)-*N*-acetylglucosamine enolpyruvyl transferase (MurA) binds UDP-*N*-acetylglucosamine (UDP-GlcNAc) and phosphoenolpyruvate (PEP) to catalyze the formation of UDP-GlcNac-3-O-enolpyruvate, a peptidoglycan precursor (Figures 1.5 and 1.6);[149] this is the first committed step in the cytoplasmic stage of peptidoglycan biosynthesis. As is the case for most enzymes involved in peptidoglycan biosynthesis, MurA is highly conserved among bacteria, is essential for cell survival, and has no human homolog.[159] Fosfomycin acts as an analogue of PEP by mimicking G3P and/or G6P, binding and blocking MurA by covalent bonding, specifically a thioether bond, between the highly strained epoxide ring of the antibiotic and a cysteine residue (Cys115) in the active site of MurA; this inhibits peptidoglycan synthesis.[149 160,161] X-ray crystallography of *E. coli* MurA complexed with its substrate, UDP-GlcNAc, and fosfomycin revealed a strong electrostatic interaction between three conserved positively charged residues of MurA and the phosphate group of fosfomycin.[162] The inactivation of the MurA gene by fosfomycin, and thus inhibition of peptidoglycan synthesis, is lethal in a variety of bacteria. It results in a decrease of cell wall content, accumulation of the nucleotide precursors, reduced growth and ultimately cell lysis and death (Figures 1.5).[149,163,164] Since the mechanism of action is different to any other antibiotic (it is the only antibiotic currently in clinical use that targets a Mur enzyme), and it differs from other compounds in general chemical structure, cross-resistance is considered unlikely.[32,163] This lack of cross-resistance has allowed fosfomycin to retain activity against many Gram-negative and Gram-positive bacteria, including MDR bacteria (see Section 1.2.4). [26,52,56]

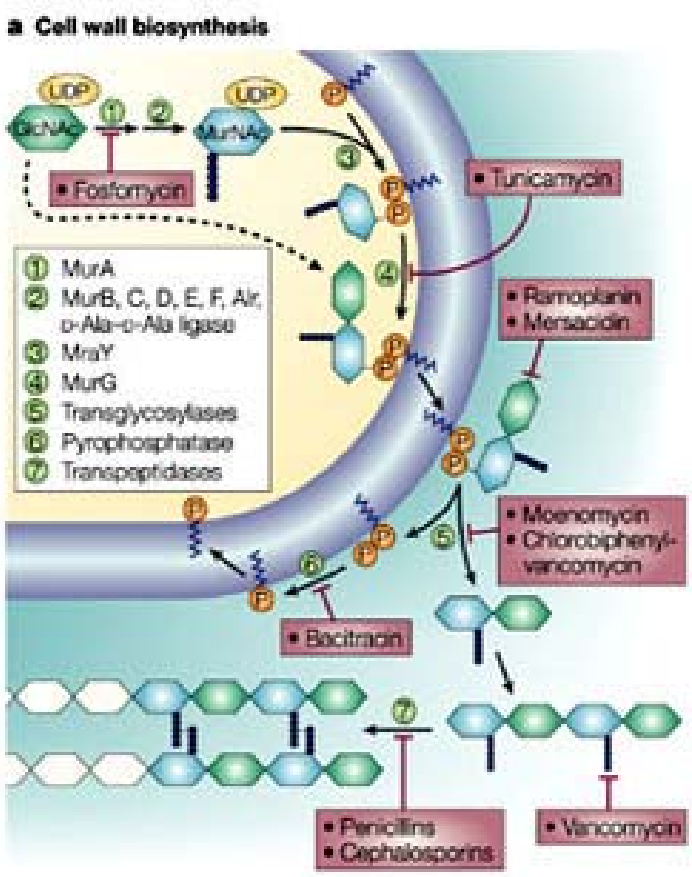

Figure 1.6. Simplified scheme of biosynthetic steps in bacterial cell wall synthesis, indicating site of inhibitor activity of fosfomycin and other cell wall inhibitors. UDP, uridine diphosphate; P, bactoprenol-phosphate (a lipid carrier for peptidoglycan units). Figure adapted from Walsh,[165] with permission.

Mechanism of resistance: Resistance to fosfomycin can occur through a number of mechanisms including reduced antibiotic uptake into bacterial cells,[149,166-168] modifications of the site of action,[161,166,169] and inactivation of the antibiotic.[170-172]

(i) Reduced permeability. As the MurA enzymes upon which fosfomycin acts are located in the cytoplasm, fosfomycin uptake is essential for antibiotic activity. A common mechanism of resistance to fosfomycin is due to genetic mutation in one or both of the chromosomally encoded transport systems, GlpT and/or UhpT (discussed above), which allow fosfomycin to enter bacterial cells, reducing uptake of fosfomycin into cells.[72,110] In *P. aeruginosa*, only mutations in the *glpT* gene influences resistance since GlpT permease is present in *P. aeruginosa* while UhpT permease is absent.[72] For *P. aeruginosa, glpT* mutations have been shown in a mouse lung infection model to be the primary cause of fosfomycin resistance *in vivo*.[70] Mutations in any of the structural genes of those pathways produce a decrease in antibiotic uptake, conferring different levels of fosfomycin resistance.[72] Some mutations result in complete inactivation of the structural genes (by frameshifts), preventing expression of the transporter, whereas others result in the production of transporters that operate less efficiently.[70,158] In a number of bacterial species (*E. coli*, *K. pneumonia* and *Proteus mirabilis*), such mutants display reduced virulence represented by lower adhesion to epithelial cells.[173-175] However, in *P. aeruginosa* disruption of the *glpT* gene does not appear to exert a significant burden on fitness or virulence.[70]

(ii) Modification of MurA. Fosfomycin exerts its action by binding to MurA and, as a consequence, modification of MurA may result in the acquisition of fosfomycin resistance.[161,176] It has been shown that a Cys115 to aspartic acid (Asp) mutation in the fosfomycin-binding site in MurA (Cys 115) produce fosfomycin resistance in *E. coli*.[161] An Asp residue is also found in the catalytic site of MurA in bacteria intrinsically resistant to fosfomycin such as *Mycobacterium tuberculosis* and *Chlamydia trachomatis*.[176,177] Overexpression of MurA in *E. coli* results in increased fosfomycin resistance with a low fitness cost.[178]

(iii) Antibiotic modification. Fosfomycin may be inactivated by catalytic opening of the epoxide ring (Figure 1.2, Section 1.2.2).[170,179,180] Three enzymes may be involved in this chemical

inactivation of fosfomycin in pathogenic bacteria – FosA, FosB and FosX; the fosfomycin resistance proteins (FosA, FosB and FosX) belong to the divalent metal-ion dependent metalloenzyme superfamily.[181] These enzymes have an extremely homologous sequences, which allows different fosfomycin enzyme genes to recombine via homologous recombination and subsequently produce enzymes that confer fosfomycin resistance.[182] However, despite their similar sequences each enzyme uses different chemical mechanisms, with different substrates, to add chemical groups to fosfomycin.[182]

FosA is a plasmid-encoded enzyme.[183] The gene *FosA* encodes a Mn^{2+}-dependent glutathione *S*-transferase which adds glutathione to the epoxide ring of fosfomycin, thus inactivating the antibiotic (Figure 1.7).[170] The *FosB* gene, found in plasmids and chromosomes,[184] encodes a non-metal dependent L-cysteine thiol transferase which inactivates fosfomycin by adding L- cysteine to the epoxide ring (Figure 1.7).[179] The *FosX* gene is located chromosomally.[180] *FosX* encodes a Mn^{2+}-dependent epoxide hydrolase which inactivates fosfomycin via the addition of H_20 to the epoxide ring (Figure 1.7).[180]

ATP is required for Fos enzyme activity.[172] A unique resistance mechanism identified in *P. aeruginosa* involves non-transferrable ATP-dependence.[185] Shimizu *et al.* found that two fosfomycin-resistant *P. aeruginosa* isolates were inactivated by fosfomycin in the presence of ATP, but not in the absence of ATP.[185] The mechanism(s) underpinning this resistance were not examined.

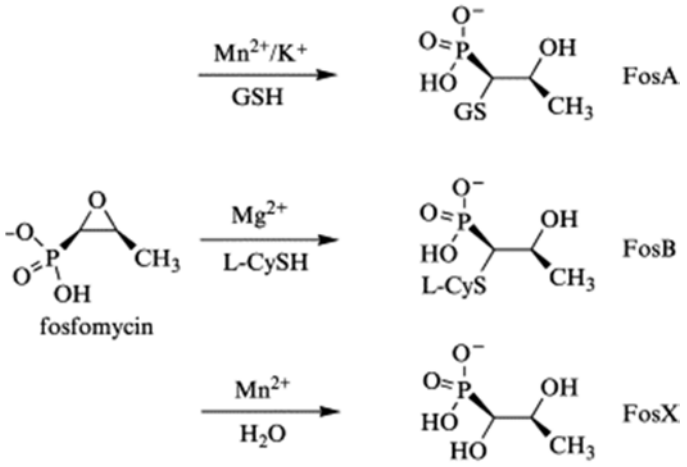

Figure 1.7. Reactions catalysed by the fosfomycin resistance proteins FosA, FosB and FosX. Figure reproduced from Rigsby *et al.,*[181] with permission.

Clinical resistance: Despite various potential mutations which may result in resistance to fosfomycin, clinical resistance rates are currently much lower than those observed *in vitro*.[32,69,110,186,187] In a number of bacterial species this may be due to the biological cost of the mutations, such as decreased growth rate and low adherence to epithelial cells, which reduce the virulence of fosfomycin-resistant bacteria.[69,70,173,188] However, as previously mentioned fosfomycin resistance caused by mutations in the *glpT* gene do not exert a significant burden on fitness or virulence of *P. aeruginosa*.[70] Another factor may be the rare clinical occurrence of changes to MurA, the target site of fosfomycin's antibacterial action, which would result in fosfomycin resistance.[72] Additionally, plasmid-encoding inactivating enzymes appear to have little impact on overall fosfomycin resistance rates in clinical isolates.[72] Currently low levels of fosfomycin use may also contribute to lower than expected clinical resistance. Worryingly, as the use of fosfomycin in the clinic has started to increase so too have reports of fosfomycin resistance in a number of bacterial species, including *P. aeruginosa*.[97,189,190] For example, the acquisition of fosfomycin resistance in a previously circulating CTX-M-15-producing urinary isolate of *E. coli* coincided with increased use of fosfomycin by 50% in a

community healthcare center in Spain.[189] In a German study conducted across two certified cystic fibrosis centers, the percentage of *P. aeruginosa* isolates (non-mucoid and mucoid) resistant to fosfomycin increased from 58.8% (*n* = 136) and 47.9% (*n* = 48) in 2001 to 74.4% (*n* = 209) and 78.8% (*n* = 118) in 2011.[97] As use of fosfomycin i.v. continues to increase such observations underpin the importance of better understanding the PK and PD of fosfomycin in order to optimize its use, including minimizing the emergence of fosfomycin resistance.

1.3 Pharmacodynamics of fosfomycin

General pharmacodynamics: The activity of an antibiotic has traditionally been quantified in terms of the MIC – the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation.[191] The MIC is a measure of potency of an antimicrobial agent and varies depending on the antibiotic and the organism.[191]

Despite MIC being utilized as a measure of potency, the MIC, which is determined at a fixed point in time, provides no information on the time-course of activity of the antibiotic, the impact of increasing antibiotic concentrations, or persistent suppression of bacterial growth following exposure to an antimicrobial (the latter known as the post-antibiotic effect [PAE]).[192,193] The impact of increasing antibiotic concentrations and the presence or absence of a PAE better describe the time-course of antimicrobial activity than the MIC.[192] As such, antibiotics are typically characterized in one of three ways: concentration-dependent killing with prolonged persistent effects, time-dependent killing with minimal-to-modest persistent effects, and time-dependent killing with prolonged persistent effects.[192] Time-dependent activity is characterized by saturation of the rate of killing with increasing concentrations, with higher concentrations not producing faster or more extensive killing than lower concentrations.[194] Concentration-dependent activity is characterized by a greater rate and extent of killing with higher concentrations over a wide concentration range. Figure 1.8 shows the effect of increasing concentrations on the *in vitro*

antimicrobial activity of three different antibiotics against a strain of *P. aeruginosa*. Increasing concentrations of tobramycin (an aminoglycoside) and ciprofloxacin (a fluoroquinolone) produced more rapid and extensive bacterial killing, whilst a similar effect for ticarcillin (a β-lactam) was only seen when the concentration was increased from 1 to 4× MIC; higher ticarcillin concentrations (4 to 64× MIC) resulted in earlier initiation of bacterial killing, but did not increase the rate of killing after 2 h.[195]

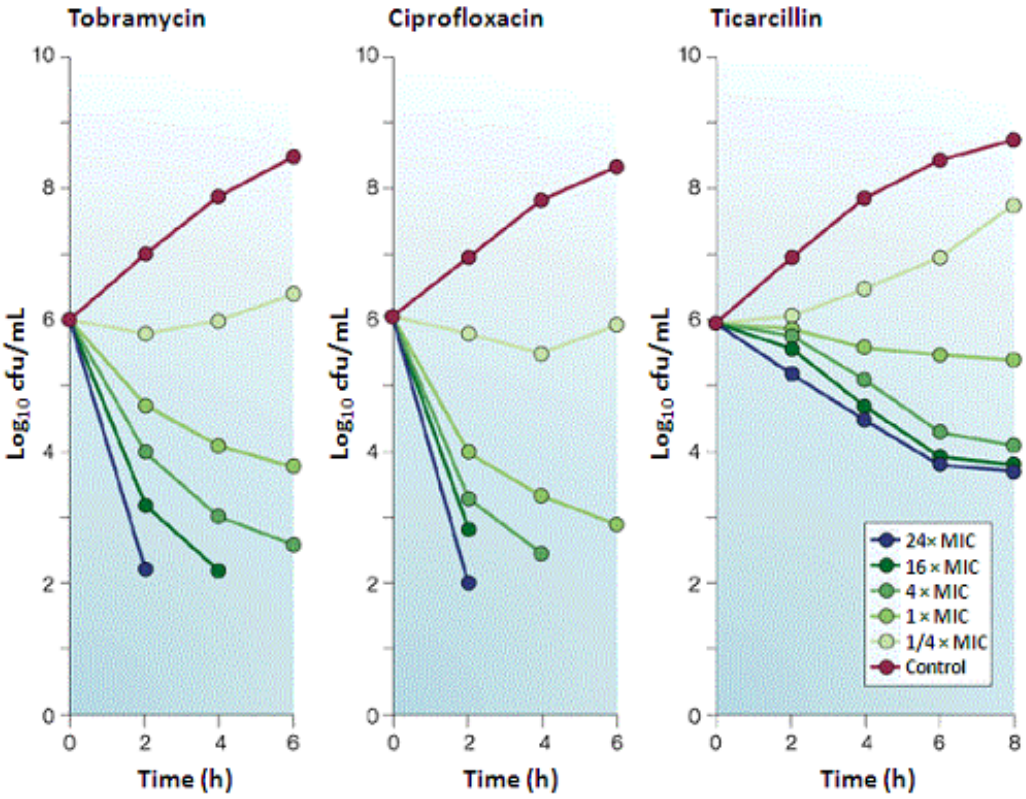

Figure 1.8. Bacterial killing against *P. aeruginosa* exposed to tobramycin (left), ciprofloxacin (centre), and ticarcillin (right) at concentrations from ¼ to 64× MIC. Data from Craig.[195] Figure adapted from Drusano,[196] with permission.

Pharmacodynamics of fosfomycin: The current CLSI and EUCAST MIC interpretive standards for fosfomycin are presented in Table 1.2 (Section 1.2.4). Currently, neither organisation provides breakpoints for *P. aeruginosa*. The CLSI requires MIC determinations for fosfomycin be carried out using agar dilution or disk diffusion, whilst the use of broth dilution is specifically recommended against.[105] This is due to the relatively high likelihood of spontaneous mutations with fosfomycin monotherapy.[25] Additionally, as G6P is required for induction of the transport system necessary for fosfomycin entry into bacterial cells (Section 1.2.6), it is recommended that G6P is added to *in vitro* testing media at a concentration of 25 mg/L.[105,197] The effect of adding G6P appears to vary between bacterial species, with Detter *et al.*[198] reporting that after the addition of G6P to Mueller-Hinton agar the MIC decreased by more than eight-fold in *E. coli* and *Klebsiella* species, but only up to two-fold in *P. aeruginosa*. EUCAST makes no recommendation for the use of agar or broth dilution when performing MIC testing with fosfomycin, however the medium used must be supplemented with 25 mg/L G6P.[106] In the literature G6P (typically at 25 mg/L) is also routinely added to media for time-kill studies involving fosfomycin.[199,200]

Significant variation exists between studies with regards to the MIC testing methods used and susceptibility breakpoints applied. Falagas *et al.*[51] detailed 23 microbiological studies examining the susceptibility of MDR non-fermenting Gram-negative bacilli to fosfomycin. The study revealed the use of four different testing methods (agar dilution, disk diffusion, broth microdilution, and Etest). Eight of 23 (35%) studies defined susceptibility based upon the CLSI definitions, 5 of 23 (22%) used other definitions, and the remaining 10 (43%) did not report which definition was employed.[51] Importantly, results may vary between the susceptibility testing method employed. For example, De Cueto *et al.*[53] tested ESBL *E. coli* and *K. pneumoniae* using agar dilution, broth microdilution, and disk diffusion. For *E. coli* the three testing methods provided equivalent results, whereas for *K. pneumoniae* the results of the three methods differed.

Perdigão-Neto *et al.*[201] determined MIC values for 15 *P. aeruginosa* isolates via both agar dilution and Etest methods. Using the CLSI breakpoints 7% and 80% of isolates were susceptible and resistant, respectively, based upon agar dilution; the equivalent values for Etest were 33% and 7%. Most recently Hirsch *et al*.[186] reported 89.5% and 93.8% categorical agreement (susceptible or resistant), respectively, between MIC results derived from Etest and disk diffusion methods compared with agar dilution; isolates of *Enterobacteriaceae, enterococci,* and *P. aeruginosa* were included. The heterogeneity of MIC testing methods present in the literature and lack of agreed-upon breakpoints makes interpretation of reported susceptibility rates difficult, especially for *P. aeruginosa*. It will not be possible to determine breakpoints for *P. aeruginosa* until appropriate clinical PK and PD data is available.

One further problem complicating the interpretation of MIC values for fosfomycin is the presence of heteroresistance (the presence of resistant subpopulations within an isolate that is susceptible based upon its MIC). Fosfomycin heteroresistance has previously been reported only in *Streptococcus pneumoniae*.[202] In Chapter 2 of this thesis, we examine whether fosfomycin heteroresistance exists in *P. aeruginosa*. It is not known whether heteroresistance reflects genetic heterogeneity in a mixed population or genetically identical cells that express different gene sets in response to divergent regulatory proteins.[203,204] The presence of heteroresistance would support the need for rational fosfomycin combination therapies (discussed in Chapter 2).

There is confusion in the literature as to whether fosfomycin displays time- or concentration-dependent activity. For example, Parker *et al*.[71] state that bacterial killing by fosfomycin is categorized pharmacodynamically as time-dependent, whereas Perdigão-Neto *et al*.[201] state that it has not been established if fosfomycin activity is concentration- or time-dependent. Time-dependent killing by fosfomycin has been reported against *Staphylococcus aureus*, although only a single isolate was employed in each study (Figure

1.9).[79,200,205,206] In contrast, Mazzei *et al*.[199] reported concentration-dependent activity against a single strain each of *Escherichia coli* and *Proteus mirabilis* (Figure 1.10). However, in that study higher concentrations did not result in faster or more extensive bacterial killing than did lower concentrations and the data would appear to show time-dependent, not concentration-dependent, killing. Interestingly, although a number of other papers are often cited in the literature in support of concentration-[207] or time-dependent[135] killing against various organisms, these studies did not examine concentration- or time-dependent activity and the authors made no claims to this effect. For *P. aeruginosa* only one study has previously reported the PD activity of fosfomycin to be time-dependent (Figure 1.11).[79] In that study, only a single reference strain (ATCC 27853) was used. In Chapter 2 of this thesis we examine whether fosfomycin displays concentration- or time-dependent activity against *P. aeruginosa* using a larger number of isolates (including both MDR and non-MDR isolates) and with both static and, for the first time (to the best of our knowledge), dynamic concentrations.

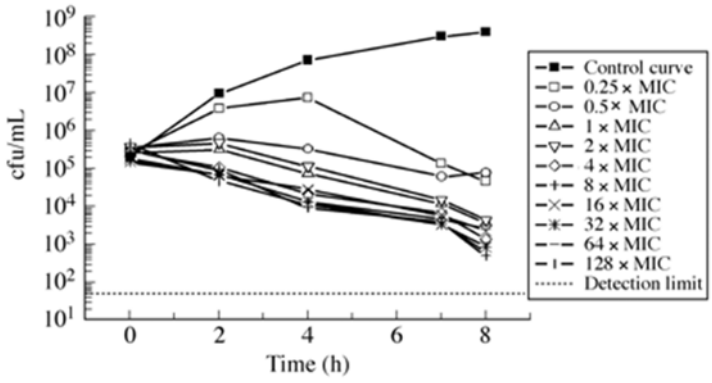

Figure 1.9 Time-kill curves for fosfomycin against *S. aureus* ATCC 29213 strain, which was exposed to different fosfomycin concentrations over a period of 8 h. Figure reproduced from Pfausler *et al.*,[200] with permission.

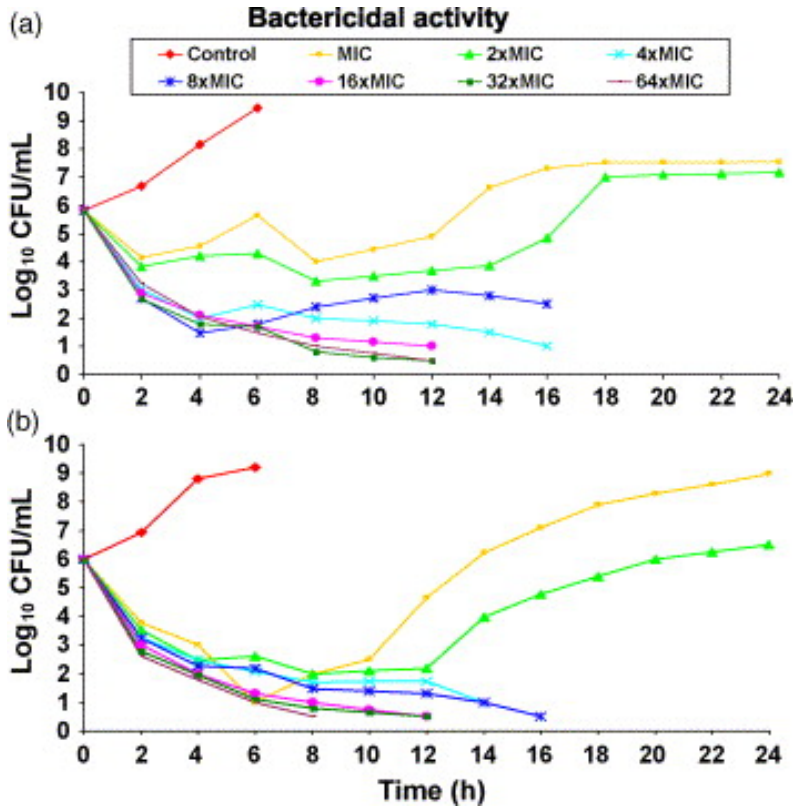

Figure 1.10. Time-kill curves for fosfomycin tromethamine against *E. coli* isolates (a) LC405 (MIC = 8 mg/L) and (b) LC406 (MIC = 8 mg/L). CFU, colony-forming units; MIC, minimum inhibitory concentration. Figure reproduced from Mazzei *et al*.,[199] with permission.

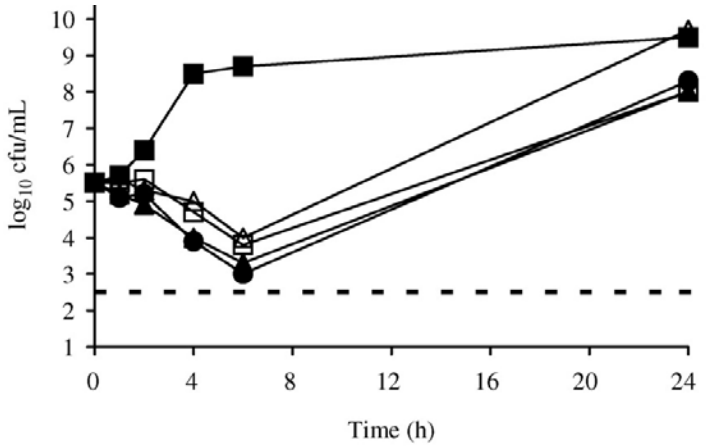

Figure 1.11 Time-kill curve for fosfomycin against *P. aeruginosa* ATCC 27853. Antibiotics were tested at concentrations of 1× MIC (open triangles), 4× MIC (open squares), 8× MIC (filled triangles) and 16× MIC (filled circles). Closed squares, no drug; broken line, bactericidal line. Figure adapted from MacLeod *et al.,* [79] with permission.

Prior to work undertaken in Chapter 2 of this thesis, we are not aware of any studies that have directly investigated the effect of inoculum on fosfomycin activity against any bacterial species. The inoculum effect is a phenomenon whereby the relative amount of antibiotic needed to inhibit bacterial growth increases with increasing bacterial density.[208] Bacterial killing by some antibiotics is known to be attenuated at high (~10^8 cfu/mL) compared to low (~$10^{5\text{-}6}$ cfu/mL) initial inocula, including against *P. aeruginosa*.[209-213] This has important implications in the clinical setting, whereby higher doses and/or combination regimens may be required to treat infections with high bacterial burden such as cystic fibrosis lung infections.[214]

Fosfomycin possess at best a moderate PAE. Mazzei *et al.*[199] examined the PAE of fosfomycin using a single clinical strain each of *E. coli* and *Proteus mirabilis*. At concentrations

of 0.25×, 1×, 4× and 8× MIC (maximum fosfomycin concentration used, 512 mg/L), the duration of the PAE ranged from 3.35 – 4.20 h in *E.coli* and 3.16 – 4.70 h in *Proteus mirabilis*. Shorter PAEs of 1.3 h, 1.0 h and 1.0 h, respectively, were reported by MacLeod *et al.*[79] using a single reference strain for each of *S. aureus, P. aeruginosa* and *E. coli* at a concentration of 2× MIC. Against *P. aeruginosa*, the actual concentration of fosfomycin used in this study was 8 mg/L. As will be discussed in Section 1.6, this concentration is very much at the low end of concentrations which are achievable in patients following i.v. administration. Prior to work reported in Chapter 2 of this thesis, this was the only study to investigate the PAE of fosfomycin against *P. aeruginosa*. Most recently, Kunakonvichaya *et al*.[215] reported a PAE of 1 h against carbapenem-resistant *P. aeruginosa*. However, the method used was not reported and it is unclear how many isolates were used.

1.4 Pharmacokinetics of fosfomycin

General pharmacokinetics: Pharmacokinetics relates to drug disposition (absorption, distribution, metabolism, and excretion) (Figure 1.12); these factors determine the time course of drug concentrations in serum and tissues for a given dosage regimen.[192] PK properties can vary significantly between various groups such as healthy volunteers, patients with cystic fibrosis and the critically ill.[216,217] As such PK data from specific patient groups is important for designing *in vitro* and clinical studies as well as for clinical practice.

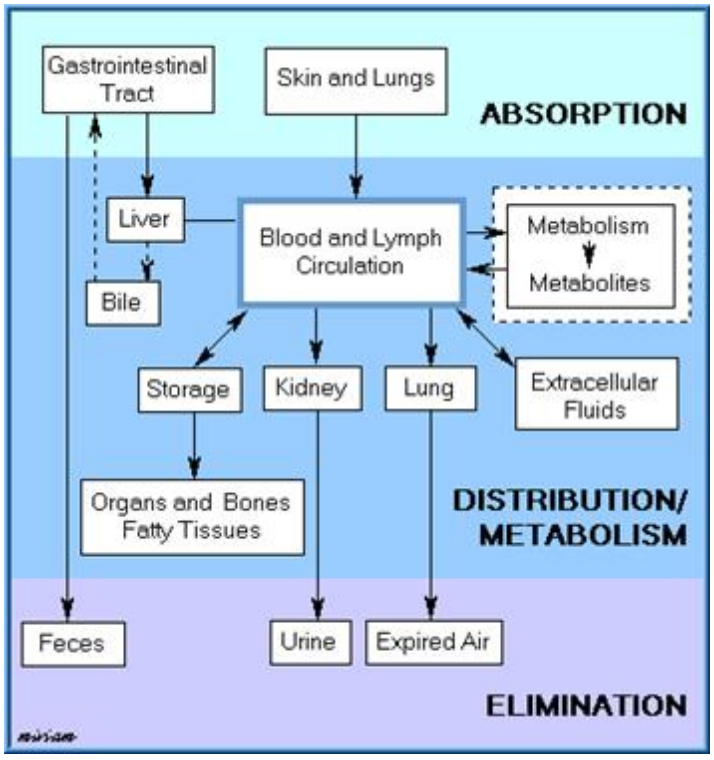

Figure 1.12. An overview of absorption, distribution, metabolism, and excretion/elimination (PK). Figure reproduced from McShane,[218] with permission.

Pharmacokinetics of fosfomycin: There is a dearth of information on the PK of fosfomycin, with direct comparison between studies complicated by the variations in drug salt form administered, route of administration, and dosages used (see Sections 1.2.2 and 1.2.3). Existing studies generally involve only a small numbers of patients (typically between 5 and 26). Importantly, as most studies have been conducted using healthy volunteers, patient factors such as impaired renal function, influence of other medications or disease states which could potentially alter PK parameters are generally absent. As fosfomycin is increasingly used in critically ill patients and patients with cystic fibrosis, the paucity of PK data in these patient groups

is of particular concern given the PK of many medications in such patients are known to differ substantially from those in healthy volunteers.[219,220] Consequently, further information on the

disposition of fosfomycin will be essential for optimizing therapeutic use to increase the efficacy and prolong the lifespan of this antibiotic. As the focus of this thesis is on the systemic use of fosfomycin, primarily for use in critically ill patients, the PK of oral fosfomycin will be considered only briefly.

As discussed in Section 1.2.5.1, fosfomycin is most commonly administered orally (as fosfomycin tromethamine) for treatment of UTIs. Fosfomycin is rapidly absorbed in the small intestine following oral administration.[221,222] In both animals and humans, absorption occurs via a saturable carrier-mediated process through a sodium-phosphate co-transporter, as well as a non-saturable process of passive diffusion following first-order kinetics.[222,223] The oral bioavailability of fosfomycin tromethamine is between 30 – 37%.[75] For fosfomycin calcium, oral bioavailability is between 12 – 28%.[224,225] The calcium salt has relatively low oral bioavailability compared to the tromethamine salt due to hydrolysis in gastric acid.[226] Following a single oral dose in patients of 3 g of fosfomycin tromethamine (the usual oral dose), the mean (± standard deviation) maximum serum drug concentration (C_{max}) of 26.1 (± 9.1) mg/L was reached within 2 h in fasting conditions, with a lower C_{max} of 17.6 (± 4.4) mg/L within 4 h following administration with a high fat meal.[75] Mean urine concentrations of 706 (± 466) mg/L were achieved within 2 – 4 h when fosfomycin tromethamine 3 g was administered as a single dose in a fasting state, and 537 (± 252) mg/L within 6 – 8 h following administration with a high fat meal; although elimination occurred more slowly following administration under fed conditions, the cumulative urinary elimination was the same under both conditions.[75] Concentrations in urine of 10 mg/L can persist for 72 – 84 h following a single 3 g oral dose.[75] The apparent V_d at steady state of the tromethamine salt in healthy volunteers was 136.1 ± 44.1 L.[76,90,163] Whether administered orally or i.v., elimination from the human body occurs almost exclusively via glomerular filtration[140] and total clearance of fosfomycin is highly correlated with the glomerular filtration rate (as measured by creatinine clearance).[64] Following oral administration 30 – 60% of the total dose of the tromethamine salt, and 9 – 18% of the calcium

salt, are excreted unchanged via the kidneys.[227] The mean half-life ($t_{1/2}$) for elimination of fosfomycin tromethamine following oral administration is 5.7 (± 2.8) h.[75]

There is limited data available regarding the PK of fosfomycin disodium, the only salt form of fosfomycin administered i.v.. Two early studies demonstrated that at steady state clearance from serum following i.v. administration was not significantly greater than from urine, indicating that fosfomycin is not metabolized and that renal excretion is probably entirely by glomerular filtration.[228,229] This was subsequently confirmed in a study involving 14 samples from healthy volunteers where renal clearance averaged 113 mL/min, approximately that of creatinine clearance.[230] Following i.v. administration of a single 4 or 8g dose of fosfomycin in 6 healthy volunteers, the mean (± standard deviation) C_{max} was 202 ± 20 mg/L and 395 ± 46 mg/L, respectively;[231] the V_d and $t_{1/2}$ were not reported. An important finding of this study was that interstitial tissue levels of fosfomycin are largely comparable to corresponding free concentrations in plasma or serum. The V_d at steady state in healthy volunteers has been reported to range from ~12 – 22 L.[225,230,232] Thus, like most hydrophilic drugs, fosfomycin has a V_d consistent with extracellular body water (~ 0.3 L/kg) in healthy volunteers.[64] The $t_{1/2}$ is typically between 2.2 – 3.4 h.[224,225]

A small number of studies have examined the PK of fosfomycin in critically ill patients.[94,135,200,233-236] Pfausler *et al*.[200] examined fosfomycin PK in 6 critically ill patients with severe haemorrhagic infarction (n = 1) or acute subarachnoid haemorrhage (n = 5). Fosfomycin was administered at a dose of 8g i.v. three times daily (infused over ~30 min). Concentrations of fosfomycin in the cerebrospinal fluid (CSF) were approximately four-fold lower in comparison with plasma (the mean ratios of the area under the concentration-time curve (AUC_8) across an 8 h dosage interval for CSF to the AUC_8 for plasma were 0.23 ± 0.07 and 0.27 ± 0.08 after single and multiple doses, respectively). The concentration-time course of fosfomycin in plasma as well as

for CSF was not significantly different after single and multiple doses (i.e. at steady-state) (Figure 1.13). After a single dose, the plasma C_{max} and $t_{1/2}$ of fosfomycin were 260 ± 85 mg/L and 3.0 ± 1 h, respectively, whereas at steady-state the equivalent values were 307 ± 101 mg/L and 4.0 ± 0.5 h. Thus, peak concentrations only increased by approximately 18 – 40% in plasma and CSF at steady state compared with single dose administration. Plasma clearance (Cl), V_d and $t_{1/2}$ were, respectively, 7.4 ± 2.3 L/h, 30.8 ± 10.2 L and 3.0 ± 1.0 h after the first dose and 5.0 ± 2.0 L/h, 26.3 ± 9.7 L and 4.0 ± 0.5 h after multiple doses. Two separate studies involving a small number of patients with sepsis investigated the PK of fosfomycin over 4 h following a single dose (either 4 or 8 g i.v. administered over 20 or 30 min).[135,233] For plasma, the PK parameters obtained in both studies were very similar to those obtained by Pfausler *et al*.[200] following a single dose. Across these three studies, the $t_{1/2}$ in plasma ranged from ~2.5 – 4h. Given the $t_{1/2}$ reported by Pfausler *et al*.[200] increased with multiple dosing, and the lowest value of 2.5 h (reported by Matzi *et al*.[233]) was determined after a single dose, the $t_{1/2}$ in critically ill patients may be longer than that observed in healthy volunteers.

In a very recent study, Parker *et al*.[235] administered a dose of fosfomycin 6 g i.v. 6-hourly to one critically ill patient. Multiple blood samples were taken over 6 h following administration of the first and fifth doses. The plasma concentration-time profile obtained is shown in Figure 1.14. The C_{max} following the initial dose was 222 mg/L, with a trough concentration (C_{min}) of 141 mg/L. Increased plasma concentrations were observed after the fifth dose, with a C_{max} of 868 mg/L and C_{min} of 592 mg/L. The accumulation of fosfomycin following multiple doses observed in this study is in stark contrast to the earlier study by Pfausler *et al*.[200] (Figure 1.13), where differences in C_{max} were much smaller following single and multiple administrations. Additionally, the C_{max} achieved was at least twice that previously reported in both healthy volunteers and critically ill patients. The $t_{1/2}$ was not reported.

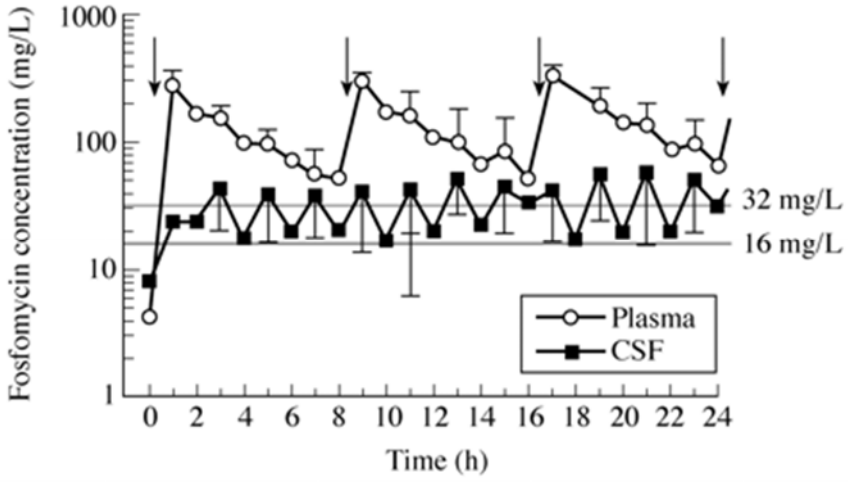

Figure 1.13. Concentration-time profiles of fosfomycin for plasma and cerebrospinal fluid (CSF) after single and multiple i.v. doses (represented by the vertical arrows) of 8g infused over 30 minutes in 6 neurointensive care patients. The solid horizontal lines represent the MIC values for pathogens. Data are shown as mean ± standard deviation. Figure reproduced from Pfausler *et al.*,[200] with permission.

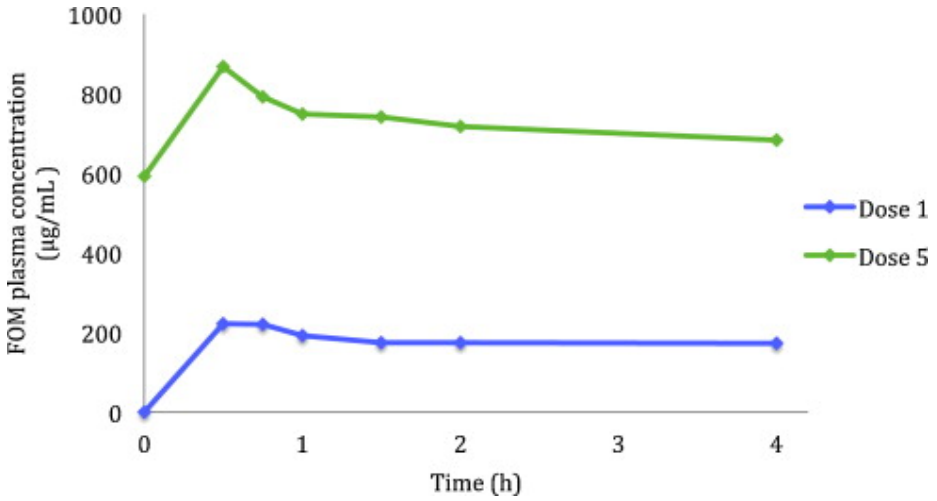

Figure 1.14. Plasma concentration-time profiles of fosfomycin in a critically ill patient receiving a 6g fosfomycin i.v. dose every 6 h, for the first and fifth doses. Figure reproduced from Parker *et al.*,[235] with permission.

Currently, there is no published data on the PK of fosfomycin following i.v. dosing in patients with cystic fibrosis. Differences in PK parameters between healthy volunteers and patients with cystic fibrosis have previously been reported for other antibiotics.[220] It will be vitally important to gather fosfomycin PK data from this patient group to guide appropriate dosing.

Plasma protein binding of fosfomycin is independent of its concentration and ranges around 0 – 2% in humans and animals.[64,237-239] As outlined earlier, *in vivo* studies have shown that equilibrium between plasma and interstitial fluid is achieved soon after administration and that interstitial tissue levels of fosfomycin are largely comparable to corresponding free concentrations in plasma or serum.[135,231] Fosfomycin has been found to distribute well into tissues such as the kidneys,[75,240,241] bladder wall,[75] prostate,[75,242,243] lungs,[233] inflamed tissues,[127,244] skeletal muscle,[135] bone,[128] heart valves,[245] cerebrospinal fluid,[200,244] and abscess fluid.[246] A review conducted by Roussos *et al*.[33] compared AUC values for plasma and various tissues and found that

penetration appears to be higher in subcutaneous and muscle tissue (AUC ratio range, site:serum, 0.48 – 0.76) compared to brain/CSF and bone tissue (AUC ratio range, site:serum, 0.08 – 0.43). The apparent high penetration of fosfomycin into many tissue types, some traditionally considered to be associated with low penetration, supports the potential use of fosfomycin in many difficult-to-treat infection sites.

1.5 Fosfomycin in combination with other antibiotics

Combination therapies with two, and sometimes three, antibiotics are often used in clinical practice to improve and broaden antimicrobial activity, minimize/eliminate the emergence of resistance, and minimize adverse effects through the use of lower doses of individual antibiotics.[247,248] Occasionally, combinations provide a protective effect against toxicity from one of the agents.[144] Though beyond the scope of this thesis, on this latter point it has been suggested a potential benefit of combination therapy with fosfomycin is its ability to protect against nephrotoxicity associated with the aminoglycoside[144,249] and glycopeptide[146] antibiotics, the antifungal agent amphotericin B,[250] as well as the chemotherapeutic agent cisplatin.[251] Combination therapies are not without risk, however, such as the possibility of increased toxicity and emergence of resistance to both antibiotics.[70,252]

As outlined in Section 1.2.5.1, the major consideration against the use of fosfomycin for systemic infections has been the perceived risk of resistance emerging during therapy, related to a high mutation frequency observed *in vitro* (although this may be less frequent *in vivo* than *in vitro*).[52,70,110,137,253] It is likely for this reason that fosfomycin is generally administered in combination with other antibiotics for systemic infections.[26,70,253] It has been suggested this relatively high mutation rate may be overcome with combination therapy, which may also potentially lead to enhanced bacterial killing (most commonly expressed as synergy).[110,138] Several potential mechanisms for synergy (which may operate simultaneously) with fosfomycin have been

proposed including the combined effect of two agents with different mechanisms of antibacterial action (so-called subpopulation synergy, the process whereby one drug kills the resistant subpopulation(s) of the other drug, and *vice versa*), and disruption of the integrity of the cell wall promoting the entry of other antibiotics into the cell (so-called mechanistic synergy) (Figure 1.15).[163,254-258] Recently, it has been suggested that accumulation of superoxides in the cell due to fosfomycin may be responsible for improved killing when combined with polymyxins.[259] Potential mechanisms of synergy with fosfomycin combinations are discussed further in Chapter 3.

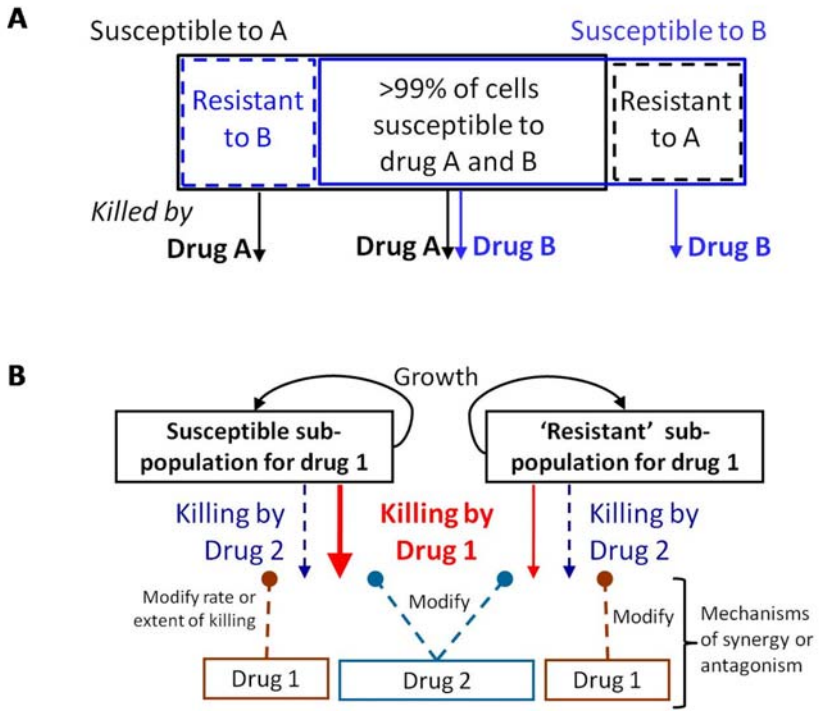

Figure 1.15. Schematic representations for subpopulation synergy (Panel A) and mechanistic synergy (Panel B). In subpopulation synergy, drug A kills the resistant subpopulations of drug B, and *vice versa*. In mechanistic synergy for drugs acting on different cellular pathways, drug A increases the rate or extent of killing by drug B, and *vice versa*. Figure adapted from Bulitta *et al.*,[260] with permission.

There is limited preclinical and clinical information examining fosfomycin combinations against and organism, and the potential advantages of fosfomycin combinations have not been systematically studied.[110] Clearly, the findings of other investigations outlined below highlight the need for systematic studies to provide essential information required for the rational design of fosfomycin combination therapy against infections caused by *P. aeruginosa*. The remainder of this section will provide an overview of both clinical and preclinical investigations of i.v. fosfomycin combination therapy.

1.5.1 Clinical studies of fosfomycin combination therapy

From the outset it should be noted that the practical and ethical considerations involved in conducting prospective studies of combination therapy in patients have resulted in major limitations with all clinical studies involving fosfomycin in combination with other antibacterial agents. Notably, the few existing studies are limited by the small number of participants, differences in dosage regimens, inclusion of combinations with numerous different antibiotics often within the one study, heterogeneity in outcome definitions (e.g. symptomatic improvement or clinical cure) and results often presented for all bacterial species and antibiotic combinations studied with no breakdown for each species or combination. Moreover, these studies typically do not stratify outcome by severity of illness, an important consideration as combination therapy is most likely to be given to the sickest patients who are more likely to die. PK data is also mostly absent. Additionally, not all studies specify whether isolates were MDR or non-MDR. Taken together, these factors preclude drawing strong conclusions from currently available clinical evidence regarding the benefit or otherwise of fosfomycin combination therapy. As a consequence, clinical studies will be considered here only briefly.

A small number of case reports and cohort studies involving fosfomycin combinations for the treatment of infections caused by *P. aeruginosa* have been published over the past decade or

so.[67,89,261-265] A retrospective cohort study from Thailand examined fosfomycin combined with either doripenem (1 g daily; *n* = 25) or colistin (5 mg/kg/day in 2 divided doses; *n* = 24) in patients with carbapenem-resistant *P. aeruginosa* pneumonia.[265] There were no statistically significant difference in either clinical or microbiological cure between the two treatment groups, with clinical cure achieved in 60% and 58% and microbiological cure in 72% and 63% of patients receiving fosfomycin-doripenem and fosfomycin-colistin, respectively; all-cause (28-day) mortality for the two treatment groups was 40% and 42%. Two prospective cohort studies undertaken in the United Kingdom examined fosfomycin combinations in patients with cystic fibrosis suffering from MDR *P. aeruginosa*-related pulmonary infections[67,261] In the study by Mirakhur *et al*.,[67] fosfomycin was used in dual and triple therapy combinations (containing one or two of: ceftazidime, colistimethate sodium, tobramycin, meropenem, aztreonam, cotrimoxazole, imipenem, piperacillin, gentamicin, or ciprofloxacin) for pulmonary exacerbations in patients with cystic fibrosis, although the number of patients who received each combination, nor what the combinations were, was not specified. In total 30 episodes across 15 patients were investigated, with fosfomycin stopped in 1 patient due to nausea. Of the remaining 14 patients all achieved clinical resolution of their chest exacerbations (as shown by a statistically significant improvement in their forced expiratory volume in 1 second [FEV_1]; pre-treatment mean 41.1 [range 14-96], post-treatment mean 49.4 [range 16-97]; *P* <0.001). A similar study by Faruqi *et al*.[261] investigated 26 pulmonary exacerbations in 7 patients. Fosfomycin (5 g i.v. 8-hourly) was used as part of a combination of two (18 courses of treatment) or three (7 courses of treatment) antibiotics (combined with ceftazidime, ciprofloxacin, colistin, gentamicin, imipenem, linezolid, piperacillin/tazobactam or vancomycin; doses not specified), with one patient receiving fosfomycin monotherapy; susceptibility to fosfomycin was not reported. The mean duration of antibiotic therapy was 14.3 days. Symptomatic improvement occurred in all patients with FEV_1 improved from an initial value of 30.9% of predicted to 34.4% of predicted following treatment.

As is obvious from the discussion above, the limited clinical data currently available does not show any significant differences in the effectiveness of fosfomycin mono- and combination therapy. The limitations associated with this data, as noted previously, also significantly hinders interpretation and, as such, the benefits to patients of fosfomycin combination therapy remain unclear. Due to the increasing threat from MDR *P. aeruginosa* infections, increasing use of i.v. fosfomycin to treat such infections, and concerns regarding the rapid development of resistance *in vitro* with fosfomycin monotherapy, further data regarding the outcomes of fosfomycin combination regimens are urgently required. Due to the practical and ethical difficulties involved in conducting such studies, preclinical investigations (both animal and *in vitro*) are vitally important for determining effective fosfomycin combination regimens.

1.5.2 Preclinical studies of fosfomycin combination therapy

1.5.2.1 Animal studies

Very few animal studies have examined fosfomycin combination therapy for treatment of infections caused by *P. aeruginosa*. Studies have been performed in rats[79,266,267] and rabbits,[268,269] with fosfomycin combined with either an aminoglycoside (tobramycin or isepamicin) or fluoroquinolone (ciprofloxacin, prulifloxacin, levofloxacin, or pefloxacin). Although animal studies are able to overcome many of the shortcomings associated with clinical studies (Section 1.6.1), major limitations nevertheless remain with currently published animal studies. Specifically, some studies do not provide reasoning for their dosage choice, and only two studies provide PK data allowing for comparisons to be made between the PK profiles achieved in the animal and human PK profiles;[268,269] such comparisons are essential to adequately assess the likely value of the combination regimen in a clinical setting. As such the remainder of this section will focus on these latter two studies.

Xiong *et al.*[269] examined the effect of fosfomycin (300 mg/kg) alone and in combination with ciprofloxacin (64 mg/kg) or pefloxacin (64 mg/kg) in a rabbit endocarditis model over 24 h against one non-MDR and one MDR isolate of *P. aeruginosa.* Therapy was initiated early (12 h after infection), when bacterial counts in aortic valve vegetations were relatively low, or late (48 h after infection), when vegetations contained a larger inoculum; all antibiotics were administered as a 24-h continuous infusion and both isolates were susceptible to fosfomycin. Steady-state serum concentrations of fosfomycin, ciprofloxacin and pefloxacin achieved were 63.9 ± 6.76 mg/L, 2.47 ± 0.47 mg/L and 4.21 ± 0.54 mg/L, respectively, and are similar to concentrations achieved in humans with standard dosing. All mono- (with the exception of pefloxacin) and combination therapy regimens significantly reduced the number of cfu/g of vegetation versus controls for the non-MDR isolate irrespective of the timing of treatment. However, against the MDR isolate only the fosfomycin-ciprofloxacin combination at the low starting inoculum reduced bacterial counts in vegetations compared with controls (bacterial counts reduced by 4.11 ± 1.13 $\log_{10}$ cfu/g of vegetation; $P < 0.001$ versus controls). In a similar study by Bugnon *et al.*,[268] fosfomycin (300 mg/kg/day, administered as a continuous infusion) and pefloxacin (109 mg/kg/day, administered with a variable flow-rate over 24 h) were examined alone and in combination in a rabbit endocarditis model at multiple inocula against two non-MDR isolates of *P. aeruginosa*. Therapy initiation was randomised, and occurred at one of four times - concurrently with bacterial inoculation, or 12- 24- or 48-h after inoculation. The steady-state serum concentration of fosfomycin achieved was 47.4 ± 11.9 mg/L, lower than that achieved by the same dose in the study by Xiong *et al*.[269] As monotherapy, pefloxacin was less bactericidal than fosfomycin. In combination against a clinical *P. aeruginosa* strain statistically significant antagonism was observed, with bactericidal activity of fosfomycin reduced but not abolished (with a 12 h pre-treatment interval, $\log_{10}$-cfu/g vegetation bacterial counts were: 4.97 [pefloxacin alone], 2.89 [fosfomycin alone] and 3.78 [combination of both antibiotics]; with a 24 h pre-treatment interval,

log_{10}-cfu/g vegetation bacterial counts were: 6.40 [pefloxacin alone], 3.32 [fosfomycin alone] and 4.94 [combination of both antibiotics]; and with a 48 h pre-treatment interval, log_{10}-cfu/g vegetation bacterial counts were: 7.35 [pefloxacin alone], 6.26 [fosfomycin alone] and 7.03 [combination of both antibiotics]. Figures were not provided for the 0 h pre-treatment interval). Additionally, as the delay between inoculation and treatment increased, the magnitude of bacterial killing by all regimens decreased. With a 12 h delay, the most effective treatment (fosfomycin monotherapy) resulted in 4.87 log_{10}-cfu/g vegetation kill difference, yet with a 48 h delay this was reduced to only 2.25 log_{10}-cfu/g vegetation kill.

Clearly, well designed animal studies examining fosfomycin combination therapy and which better simulate fosfomycin PK (namely, the peaks and troughs experienced in patients) are urgently required. Such investigations will be essential to help built on the knowledge gained from *in vitro* studies (discussed in Section 1.5.2.2) and optimize fosfomycin therapy.

1.5.2.2 In vitro studies

Methods employed for *in vitro* synergy testing most commonly include the microbroth dilution (checkerboard) and time-kill methods.[270] The checkerboard technique typically provides only inhibitory data, whereas the time-kill method measures the bactericidal activity of the combination being tested. The other major advantage of the time-kill method over the checkerboard technique is that it provides a picture of antimicrobial action over time (based on serial viable counts), whereas the checkerboard technique is usually examined at a single time point (after 16 to 24 h of incubation).[271]

In vitro studies examining combination therapy most commonly define the PD interaction of the agents in terms of additivity, synergy, indifference or antagonism.[271] Multiple definitions have been suggested for these terms which vary depending on the susceptibility testing method

used but also within each method. For the commonly-used checkerboard method synergy has traditionally been defined as a fractional inhibitory concentration index (FICI) of ≤0.5, additivity as an FICI of 1.0, and antagonism as an FICI of 2.0.[272] However, more recently it has been suggested that antagonism should be defined as an FICI of >4 to account for imprecision of the technique and because an FICI of 2.0 more likely suggests indifference rather than a true antagonistic effect.[273] The checkerboard microbroth dilution method is the most frequently used method for measuring such interactions. However, despite its widespread use, this method is less discriminatory than other more sophisticated *in vitro* methods (e.g., static or PK/PD time-kill models) for assessing the interactions of antimicrobial agents and its predictive value has been questioned.[274,275]

Time-kill models can be subdivided into static and PK/PD models. In static time-kill models (such as that used in Chapter 3), bacteria are exposed to static (fixed) concentrations of an antibacterial agent over a defined period of time, whereas in PK/PD models a dynamic concentration of drug designed to mimic *in vivo* PK is employed. In both models synergy and antagonism have traditionally been defined, respectively, as a 100-fold increase (i.e. a $\geq$2-$\log_{10}$ lower cfu/mL) or decrease (i.e. a $\geq$2-$\log_{10}$ higher cfu/mL) in killing at 24 h with the combination relative to its most active component (Figure 1.16).[271] Variations on, and additions to, these definitions abound in the literature which complicates the interpretation and comparison of results from different studies. However, based on the standard definition of synergy outlined above a combination which achieves poor overall antimicrobial activity may still be 'synergistic' given the definition does not take into account the amount of bacterial killing that has or has not occurred (Figure 1.16); the converse situation also applies for antagonism. Consequently, a rigorous analysis of combination therapy which includes regard to clinical applicability of the concentrations employed and the overall antimicrobial activity achieved is warranted.

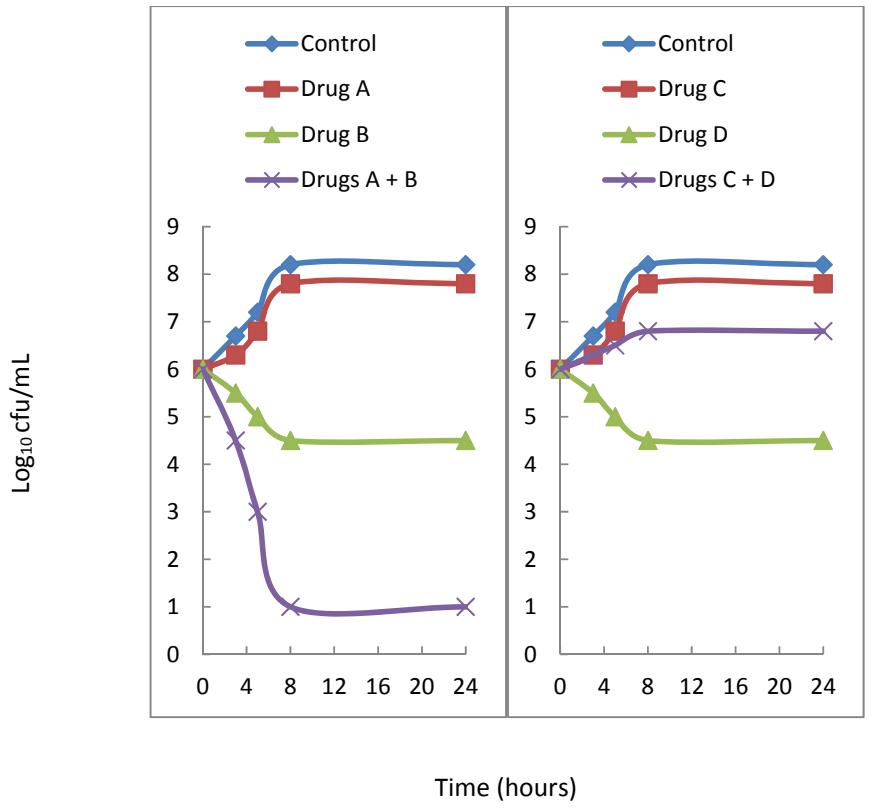

Figure 1.16. Concentration-time profiles showing the effect of antimicrobial combinations as determined in a time-kill study. Left panel (A + B), synergism; right panel (C + D) antagonism.

For fosfomycin combination therapy, the most common organisms studied are *E. coli*, *S. aureus*, and *K. pneumoniae*. Specifically for *P. aeruginosa*, existing preclinical investigations of fosfomycin combination therapy with antibiotics such as carbapenems (meropenem, imipenem, and doripenem),[49,137,215,276,277] cephalosporins (ceftazidime and cefepime),[276,277] penicillins (piperacillin),[277] aminoglycosides (tobramycin, amikacin and gentamicin),[137,276-278] polymyxins (colistin),[49,95,137] monobactams (aztreonam),[277] and fluoroquinolones (ciprofloxacin, ofloxacin and levofloxacin)[137,277,279,280] have shown good synergistic effects against MDR isolates despite many of the isolates being resistant to the second antibiotic. The majority of these studies used the less

discriminatory FICI or Etest methods against a small number of isolates to qualitatively define synergy, additivity, indifference or antagonism. The few studies that used more advanced time-kill methods generally did not appropriately capture the full concentration-effect relationship, the complete time-course of interaction, and used interpretive criteria where all synergistic combinations are deemed equal. This type of situation led to the recent urgent call for fosfomycin combination studies which employ more stringent definitions of synergy than are currently available in the literature.[281] The remainder of this Section will examine significant recent time-kill studies undertaken with fosfomycin against *P. aeruginosa*.

Only one study each has examined fosfomycin combined with a polymyxin or ciprofloxacin using time-kill methodology, and only two studies have examined fosfomycin plus tobramycin. Di *et al*.[95] examined fosfomycin-colistin combinations (each at 0.5× or 1× the MIC) against five carbapenem-resistant *P. aeruginosa* isolates (including two fosfomycin-resistant isolates) of unknown MDR status. Combinations at both concentrations resulted in bacterial eradication in almost all cases, with no viable bacteria detected at 24 h. In contrast, regrowth with all monotherapies at this time was above ~10^7 $\log_{10}$ cfu/mL. Yamada *et al*.[280] observed enhanced bacterial killing (~2 – 4-log) over 10 h with a fosfomycin-ciprofloxacin combination (each at 0.5× MIC; fosfomycin concentrations ranged from 16 – 64 mg/L) against four clinical isolates of ciprofloxacin-resistant *P. aeruginosa* (one strain fosfomycin-resistant). For tobramycin, Díez-Aguilar *et al*.[278] recently reported a fosfomycin-tobramycin combination to have virtually no beneficial effect on bacterial killing of eight *P. aeruginosa* clinical isolates (all with a fosfomycin MIC of 64 mg/L; five tobramycin-susceptible and three tobramycin-resistant). However, in that study the concentrations of fosfomycin and tobramycin employed were 90 mg/L and 10 mg/L, respectively, and tobramycin monotherapy rapidly eliminated viable bacteria against the susceptible isolates precluding any potential benefit with the combination. No benefit was

observed with the combination against the three tobramycin-resistant isolates. In contrast, MacLeod *et al*.[282] examined fosfomycin (12.8 mg/L) plus tobramycin (3.2 mg/L) against *P. aeruginosa* reference strain ATCC 27853. The combination resulted in greatly enhanced early bacterial killing compared to equivalent monotherapy, with bacterial eradication by 6 h.

Remarkably few studies have examined the emergence of resistance to fosfomycin with combination therapy against any organism. A small number of studies have examined single-step resistance to fosfomycin and its combinations against *P. aeruginosa*.[70,79,137,282,283] Two of these studies highlighted the importance of the choice of second antibiotic, demonstrating that while certain combinations of fosfomycin (e.g. fosfomycin plus tobramycin, meropenem, or ciprofloxacin) makes improbable the emergence of resistance to both antibiotics, even in hypermutable strains, other combinations (e.g. fosfomycin plus imipenem or ceftazidime) led to higher-than-expected numbers of mutants.[70,137] MacLeod *et al*.[79] examined the development of resistance of *P. aeruginosa* to fosfomycin, tobramycin, and their combination after a single exposure (4× the MIC) to the antibiotics. Although the spontaneous mutation frequency (*i.e.* the number of colonies growing on antibiotic-containing agar plates divided by the number of colonies growing on antibiotic-free agar) resulting in resistance after a single exposure to the combination was less than the frequencies of fosfomycin or tobramycin, the differences were ≤2 $\log_{10}$ relative to tobramycin alone. However, in a subsequent study by the same group the mutation frequency of a fosfomycin-tobramycin combination at 16× the MIC was ≥3 $\log_{10}$ and 4 $\log_{10}$ lower than for fosfomycin and tobramycin alone, respectively.[282] Similar results were reported by McCaughey *et al*.[283] In similar studies involving a reference strain of *P. aeruginosa* and its hypermutable derivative, Rodríguez-Rojas *et al*.[70,137] reported that the mutation frequencies of various fosfomycin combinations were typically greatly reduced compared to the individual antibiotics. These studies included combinations with tobramycin, ciprofloxacin and colistin (polymyxin E),

all of which reduced the mutation frequencies in both strains to below the limit of detection ($<1\times 10^{-10}$ or $\leq 10^{-11}$). However, the use of simple mutation frequencies carried out on agar does not capture the full concentration-effect relationship and complete time-course of emergence of resistance. In Chapter 3 of this thesis we have employed a static time-kill model to investigate for, what we believe to be, the first time the emergence of fosfomycin resistance with combination therapy over time against any organism.

Clearly, due to the need for, and potential benefits of, effective antibiotic combinations against MDR *P. aeruginosa* and the risk of resistance to fosfomycin developing if inappropriate combinations are used, well designed *in vitro* studies are required. Such studies will provide essential information to assist in the design of rational combination therapy. Chapter 3 of this thesis systematically investigates fosfomycin in combination with polymyxin B, tobramycin, or ciprofloxacin utilizing a static time-kill model. This investigation utilized clinically relevant concentrations of each antibiotic and included fosfomycin-heteroresistant isolates and an examination of the emergence of resistance.

1.6 Summary

The rapid increase of antibiotic resistance globally coupled with a lack of new antibiotics in development represents one of the three greatest threats to human health.[5] Antibiotics effective against resistant 'superbugs' are urgently required.[1,284,285] Fosfomycin is an old antibiotic which retains significant activity against many MDR Gram-negative 'superbugs' including *Pseudomonas aeruginosa*.[25,33] Fosfomycin is traditionally indicated as a single-dose oral treatment for uncomplicated UTIs,[50] but due to the growing threat from MDR 'superbugs' interest in the use of i.v. fosfomycin as an antibiotic of last resort against otherwise untreatable infections caused by

P. aeruginosa is increasing;[52,66,68] unfortunately, fosfomycin resistance may occur when used as monotherapy.[25,110] However, as fosfomycin was never subject to contemporary drug development procedures very little is known about optimal fosfomycin dosing to maximize bacterial killing and minimize the emergence of resistance.

This Masters project applies modern principles of antimicrobial PD to fosfomycin to generate essential pre-clinical information required for the rational design of optimal i.v. dosing strategies for infections caused by *P. aeruginosa*. The current level of susceptibility of *P. aeruginosa* to fosfomycin within the Australian context, and essential PD properties such as concentration- or time-dependent killing, PAE and inoculum effect are investigated in this thesis. Fosfomycin combination therapy against MDR *P. aeruginosa* is also examined.

Optimising fosfomycin administration will strengthen the armamentarium available to clinicians to treat MDR *P. aeruginosa* infections, improve clinical outcomes for critically ill patients and patients with cystic fibrosis, and prolong the therapeutic utility of this increasingly important therapeutic agent. In view of this, the specific aims of this thesis are to:

1. **Establish essential *in vitro* pharmacodynamic data of fosfomycin against *P. aeruginosa* (Chapter 2):** The *in vitro* PD properties of fosfomycin were investigated via MICs, time-kill kinetics (static and dynamic models), baseline population analysis profiles (PAPs) and PAE. Two inocula were included in static time-kill studies to examine the effect of inoculum on bacterial killing.

2. **Investigate *in vitro* bacterial killing and emergence of fosfomycin resistance with fosfomycin monotherapy and combination therapy against *P. aeruginosa* (Chapter 3):** The effects of fosfomycin-containing combinations on bacterial killing were

investigated via time-kill kinetics (static models). The effects of the combination on the emergence of fosfomycin resistance was assessed via PAPs. Fosfomycin was combined with ciprofloxacin, polymyxin B or tobramycin. Fosfomycin-susceptible (and – heteroresistant) and –resistant strains of *P. aeruginosa* were included in the studies.

1.7 Structure of thesis

As the methods employed for the studies contained within this thesis are described in detail in each chapter, a separate methods chapter has not been included. Chapters 2 and 3, (research findings) comprise manuscripts which have been published or submitted for publication.

Declaration for Thesis Chapter Two

Declaration by candidate

In the case of Chapter Two, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
• design of the study; • all laboratory experiments; • data analysis and interpretation; • preparation of the initial draft of the manuscript and subsequent revisions; and • formulation of the conclusions and hypothesis arising from the results of the study.	65

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution
Michelle McIntosh	• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation; • review of manuscript drafts and revisions; and • formulation of conclusions and hypotheses arising from the results of the study.
Anton Peleg	• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation; • review of manuscript drafts and revisions; and • formulation of conclusions and hypotheses arising from the results of the study.
Carl Kirkpatrick	• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation; • review of manuscript drafts and revisions; and • formulation of conclusions and hypotheses arising from the results of the study.
Phillip Bergen	• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation; • review of manuscript drafts and revisions; and • formulation of conclusions and hypotheses arising from the results of the study.

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 17/12/2015
Main Supervisor's Signature	[redacted]	**Date** 17/12/2015

Chapter Two

In vitro pharmacodynamics of fosfomycin against clinical isolates of *Pseudomonas aeruginosa*

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Running title: Pharmacodynamics of Fosfomycin against *P. aeruginosa*

Key words: fosfomycin, *P. aeruginosa*, heteroresistance, population analysis profiles, time-kill, post-antibiotic effect, inoculum effect

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Abstract

Background: The use of fosfomycin for treatment of systemic infections due to multidrug resistant *Pseudomonas aeruginosa* is increasing. However, pharmacodynamic data for fosfomycin is limited.

Methods: Sixty-four clinical isolates of *P. aeruginosa* (MDR and non-MDR) from two Australian hospitals were collected; fifty-nine isolates were from patients with cystic fibrosis and five from critically ill patients. The *in vitro* pharmacodynamic properties of fosfomycin (disodium) were investigated via MICs (all isolates) and, for selected isolates, via time-kill kinetics (static and dynamic models; concentration range, 16 – 1024 mg/L), population analysis profiles (PAPs), and post-antibiotic effect (PAE). Two inocula (~10^6 and 10^8 cfu/mL) were included in static time-kill studies to examine the effect of inocula on bacterial killing.

Results: MICs ranged from 1 - >512 mg/L, with 61% of isolates considered fosfomycin-susceptible (MIC ≤64 mg/L). The MIC distributions for MDR and non-MDR isolates were similar. Baseline PAPs indicated heteroresistance in all isolates tested. Time-kill studies showed moderate (maximum killing ~3-$\log_{10}$ cfu/mL), time-dependent killing at the low inoculum with regrowth at 24 h. All but the lowest concentration resulted in the complete replacement of fosfomycin-susceptible colonies by -resistant colonies. Bacterial killing was virtually eliminated at the high inoculum. The PAE ranged from 0.3 – 5.5 h.

Conclusions: These data suggest monotherapy with fosfomycin may be problematic for the treatment of infections caused by *P. aeruginosa*. Further investigation of fosfomycin combination therapy is warranted.

Introduction

In recent decades there has been a remarkable increase in resistance to currently available antibiotics and a marked decline in discovery and development of novel antibiotics, sparking fears of a return to the pre-antibiotic era.[22] This situation is particularly important for the Gram-negative bacterium *Pseudomonas aeruginosa*, where the availability of a new class of antibiotic that is able to evade known resistance mechanisms is many years away.[284,285] *P. aeruginosa* is an important pathogen in a broad range of nosocomial and community-acquired infections, with multidrug-resistant (MDR) *P. aeruginosa* identified by the Infectious Diseases Society of America (IDSA) as one of the top six pathogens threatening healthcare systems.[7,10,11] With few new antibiotics in the drug development pipeline, clinicians have been forced to reconsider the therapeutic potential of older, underutilised antibiotics such as fosfomycin for the treatment of infections caused by MDR Gram-negative organisms.[66-68] Indeed, studies have demonstrated that fosfomycin is a promising agent, especially as part of combination therapy, for the treatment of various infections caused by both MDR Gram-negative and Gram-positive organisms. [49,52,56,68] As resistance to the commonly used antimicrobials continues to increase, the focus on fosfomycin as a therapeutic alternative will increase.

Fosfomycin was first isolated from cultures of Streptomyces species in 1969.[34] Originally named phosphonomycin, it is a phosphonic acid derivative with a molecular mass of 138 Da. Its reported mechanism of action is to disrupt the formation of the peptidoglycan precursor uridine diphosphate N-acetylmuramic acid (UDP-MurNAc),[149] the first cytoplasmic step in the biosynthesis of the bacterial cell wall. This biosynthetic step is unaffected by other antimicrobials, meaning cross-resistance with other classes of antibiotics is unlikely.[163] The result is reduced peptidoglycan synthesis, reduced growth, and ultimately cell lysis.[149] Bearing no structural relationship with other current classes of antimicrobials, fosfomycin is recognised as its own class

of antibiotics. It has a broad spectrum of activity against many Gram-negative and Gram-positive bacteria as well as aerobic pathogens. Furthermore, it is generally well tolerated at therapeutic doses (typically 4 – 8 g intravenously 8-hourly for serious systemic infections), the most commonly reported adverse effect being hypokalaemia due to a high sodium load (fosfomycin disodium contains about 330 mg of sodium per gram of fosfomycin).[25,78]

Fosfomycin is available orally as fosfomycin tromethamine (aka trometamol) and fosfomycin calcium, and intravenously and intramuscularly as fosfomycin disodium.[32] Oral fosfomycin tromethamine is currently indicated for the single-dose treatment of uncomplicated urinary tract infections (UTIs) caused by *Escherichia coli* and *Enterococcus faecalis* in women.[50] However, the rapid emergence and spread of MDR pathogens including *P. aeruginosa* has resulted in renewed interest in the parenteral use of fosfomycin (disodium) to treat infections other than UTIs.[286] Indeed, fosfomycin was recently identified as one of four antimicrobials holding the greatest promise of utility worldwide for managing infections that result from many currently-resistant Gram-negative bacilli.[286] Importantly, fosfomycin was identified and developed prior to the introduction of modern drug development and approval procedures. This was a time where development occurred on a more or less trial and error basis rather than the information-rich informed approach presently undertaken.[287] Consequently, substantial gaps in our knowledge of the pharmacokinetic and pharmacodynamic properties of this antimicrobial currently exist. If known, these properties could be used to optimise the use of fosfomycin and improve patients care and outcomes. The urgency of this situation has been recently highlighted with an international call for a more advanced understanding of the pharmacokinetics and pharmacodynamics of fosfomycin to ensure optimal therapy, especially in critically ill patients.[38,71,111] Clearly, an improved understanding of the pharmacology of this antimicrobial is urgently required and will

form the basis for strategies aimed at maximizing bacterial killing and minimizing the emergence of resistance in target patient populations.

The aim of this study was to investigate the *in vitro* pharmacodynamic properties, namely minimum inhibitory concentrations (MICs), population analysis profiles (PAPs), bacterial killing (including the effect of inoculum), and the post-antibiotic effect (PAE) of fosfomycin against clinical isolates of *P. aeruginosa*, including MDR isolates. Such information is essential for future development and evaluation of pre-clinical and clinical pharmacokinetics/pharmacodynamics of fosfomycin and optimization of therapy.

Materials and Methods

Antibiotic, bacterial isolates and MIC determination

Fosfomycin disodium was purchased from Waterstone Technology (Lot: 20131012, Carmel, Indiana, USA). As the *in vitro* susceptibility of fosfomycin is greatly influenced by the presence of glucose-6-phosphate (G6P) in the culture medium,[111] Mueller-Hinton agar and cation-adjusted Mueller-Hinton broth (CAMHB) used in all experiments was supplemented with 25 mg/L G6P (Lot: SLBD7775V, Sigma-Aldrich, Castle Hill, NSW, Australia) as per CLSI guidelines.[288] Fosfomycin and G6P stock solutions were prepared in Milli-Q water (Millipore, Melbourne, Australia) and sterilised by filtration with a 0.2-μm-pore-size filter (VWR Australia, Brisbane, Australia).

Sixty-four clinical isolates and *P. aeruginosa* ATCC 27853 (American Type Culture Collection, Manassas, VA, USA) were studied (Table 2.1). Fifty-nine isolates were obtained from sputum from patients with cystic fibrosis (CF) at the Alfred Hospital (Melbourne, Australia) and included a mixture of both mucoid and non-mucoid strains. Five isolates were obtained from blood

from critically ill patients at The Royal Brisbane and Women's Hospital (Brisbane, Australia). Isolates were stored in tryptone soy broth (Oxoid, Hampshire, England) with 20% glycerol (Ajax Finechem, Seven Hills, NSW, Australia) in cryovials at -80^{o}C and subcultured onto Nutrient Agar plates (Media Preparation Unit, University of Melbourne, Melbourne, VIC, Australia) before use.

Table 2.1. Susceptibility of P. aeruginosa to fosfomycin

No	Strain[a]	Mucoid Status[b]	Fosfomycin MIC (mg/L)	Susceptible to fosfomycin[c]	MDR[d]
1	ATCC 27853	n/m	8	Yes	No
2	CW 1	n/m	8	Yes	Yes
3	CW 2	muc	64	Yes	Yes
4	CW 3	muc	128	No	No
5	CW 4	n/m	128	No	Yes
6	CW 5	n/m	128	No	Yes
7	CW 6	muc	32	Yes	Yes
8	CW 7	muc	16	Yes	Yes
9	CW 8	n/m	>512	No	Yes
10	CW 9	n/m	128	No	Yes
11	CW 10	muc	128	No	Yes
12	CW 11	muc	>512	No	Yes
13	CW 12	muc	128	No	No
14	CW 13	n/m	>512	No	Yes
15	CW 14	muc	4	Yes	No
16	CW 15	muc	64	Yes	No
17	CW 16	muc	128	No	Yes
18	CW 17	n/m	4	Yes	Yes

19	CW 18	muc	256	No	No
20	CW 19	muc	>512	No	Yes
21	CW 20	n/m	32	Yes	Yes
22	CW 21	muc	32	Yes	No
23	CW 22	n/m	8	Yes	Yes
24	CW 23	muc	64	Yes	No
25	CW 24	n/m	64	Yes	No
26	CW 25	muc	64	Yes	No
27	CW 26	n/m	16	Yes	Yes
28	CW 27	muc	32	Yes	No
29	CW 28	n/m	32	Yes	Yes
30	CW 29	muc	32	Yes	Yes
31	CW 30	n/m	>512	No	Yes
32	CW 31	muc	>512	No	No
33	CW 32	n/m	1	Yes	Yes
34	CW 33	muc	64	Yes	Yes
35	CW 34	n/m	512	No	Yes
36	CW 35	muc	>512	No	Yes
37	CW 36	n/m	64	Yes	Yes
38	CW 37	muc	32	Yes	No
39	CW 38	muc	64	Yes	No
40	CW 39	n/m	128	No	Yes
41	CW 40	n/m	128	No	Yes
42	CW 41	muc	32	Yes	Yes
43	CW 42	n/m	32	Yes	Yes
44	CW 43	muc	32	Yes	No
45	CW 44	muc	512	No	No
46	CW 45	n/m	8	Yes	Yes

47	CW 46	muc	8	Yes	Yes
48	CW 47	muc	256	No	No
49	CW 48	muc	512	No	No
50	CW 49	n/m	64	Yes	No
51	CW 50	muc	64	Yes	No
52	CW 51	muc	128	No	No
53	CW 52	muc	64	Yes	No
54	CW 53	muc	64	Yes	No
55	CW 54	n/m	32	Yes	Yes
56	CW 55	n/m	64	Yes	Yes
57	CW 56	muc	>512	No	No
58	CW 57	n/m	32	Yes	Yes
59	CW 58	muc	32	Yes	No
60	CW 59	n/m	32	Yes	No
61	CR 906	n/m	>512	No	Yes
62	CR 907	n/m	>512	No	Yes
63	CR 1005	n/m	32	Yes	Yes
64	CR 1032	n/m	64	Yes	No
65	CR 1033	n/m	32	Yes	No

[a] All isolates obtained from patients with cystic fibrosis except five isolates obtained from critically ill patients (CR 906, CR 907, CR 1005, CR 1032, CR 1033) and the reference strain (ATCC 27853).

[b] muc, mucoid; n/m, non-mucoid.

[c] S, susceptible; R, resistant. Susceptibility was defined as an MIC ≤64 mg/L and resistance an MIC >64 mg/L.

[d] Multidrug-resistant (MDR) was defined as resistance to at least 1 agent in ≥3 of the following antimicrobial classes: aminoglycosides, antipseudomonal carbapenems, antipseudomonal cephalosporins, antipseudomonal fluoroquinolones, antipseudomonal penicillins plus β-lactamase inhibitors, monobactams, phosphonic acids and polymyxins.[289]

The MIC of fosfomycin for each isolate was determined in duplicate on separate days by agar dilution on Mueller-Hinton agar (Oxoid, Hampshire, England) supplemented with 25 mg/L G6P according to CLSI guidelines. From these results the MIC range, MIC_{50} (antibiotic concentration required to inhibit the growth of 50% of the pathogens), and MIC_{90} (antibiotic concentration required to inhibit the growth of 90% of the pathogens) were determined. At present, the susceptibility breakpoints for fosfomycin against *P. aeruginosa* have not been established. The existing CLSI breakpoints for the Enterobacteriaceae are ≤64 mg/L, 128 mg/L and ≥256 mg/L for susceptibility, intermediacy and resistance, respectively.[288] For the purposes of this study we considered all isolates with an MIC ≤64 mg/L to be susceptible and >64 mg/L to be resistant. Concentrations of greater than 64 mg/L are readily achievable in plasma as well as other sites such as subcutaneous, interstitial and bone tissue at normal therapeutic doses.[33]

MDR was defined as per the definition of Magiorakos *et al*.,[289] namely resistance to at least 1 antimicrobial in ≥3 of the following antimicrobial classes: aminoglycosides, antipseudomonal carbapenems, antipseudomonal cephalosporins, antipseudomonal fluoroquinolones, antipseudomonal penicillins plus β-lactamase inhibitors, monobactams, phosphonic acids and polymyxins.

Population analysis profiles (PAPs)

The possible presence of fosfomycin-resistant subpopulations at baseline was determined via population analysis profiles (PAPs; inoculum ~$10^{8.5}$ cfu/mL). To ensure a broad selection of isolates was included, fourteen clinical isolates were randomly chosen from those isolates with MIC values (see MIC section above) ≤64 mg/L (i.e., isolates considered susceptible to fosfomycin). The reference strain ATCC 27853 was also examined for comparison. Using an automatic spiral plater (WASP, Don Whitley Scientific, West Yorkshire, UK) 50 µL of

appropriately diluted (with saline) bacterial cell suspension was distributed onto Mueller-Hinton agar (Media Preparation Unit; supplemented with 25 mg/L G6P). Plates were impregnated with fosfomycin at concentrations of 0, 8, 16, 32, 64, 128 and 256 mg/L. After 24 h of incubation (48 h for plates with small colonies) at 35ºC, colonies were counted using a ProtoCOL® colony counter (Synbiosis, Cambridge, UK). The limit of detection was 20 cfu/mL (equivalent to 1 colony per plate) and limit of quantification was 400 cfu/mL (equivalent to 20 colonies per plate) as specified in the ProtoCOL® manual. PAPs were also performed in the time-kill studies (discussed below). Fosfomycin heteroresistance was defined as the presence within a fosfomycin-susceptible isolate (i.e., MIC, ≤64 mg/L) of subpopulations able to grow on agar containing >64 mg/L fosfomycin.

Post-antibiotic effect (PAE)

PAE values for ATCC 27853 and one clinical isolate each from a patient with CF and a critically ill patient were determined by the standard *in vitro* method.[290] Each isolate (~$10^{5.5}$ cfu/mL) in logarithmic phase growth was exposed to fosfomycin in CAMHB (37ºC) supplemented with G6P (25 mg/L) for 3 hours at concentrations of 4, 32 and 256 mg/L. An exposure of 3 h was chosen due to the relatively slow killing effect of fosfomycin. Fosfomycin was then removed by twice centrifuging at 4,000 rpm for 10 min, decanting the supernatant and resuspending in pre-warmed CAMHB with G6P. Viable counts were performed hourly for 6 h on G6P-containing Mueller-Hinton agar. Growth controls were similarly performed but without exposure to fosfomycin. Colonies were counted after 24 h of incubation at 37°C as described for PAPs above. The PAE was determined by subtracting the time required for the viable counts of the growth controls to increase 1 $\log_{10}$ cfu/mL above the $\log_{10}$ cfu/mL immediately after dilution from the corresponding time for antibiotic-exposed cultures.

Time-kill kinetics and the emergence of fosfomycin resistance

To explore the antimicrobial activity of fosfomycin static time-kill studies (i.e. studies utilizing a constant concentration of fosfomycin) were performed at an initial inoculum of ~10^6 cfu/mL using four clinical isolates (CW 7, CW 17, CR 1005 and CR 1033) and ATCC 27853. The isolates employed were chosen based on MIC results to represent a spread of MIC values, strains from CF and critically ill patients, and MDR and non-MDR strains. In these studies, PAPs were additionally conducted on two strains at baseline (0 h) and 24 h on Mueller-Hinton agar impregnated with fosfomycin at concentrations of 32, 64, 128 and 256 mg/L to explore the emergence of fosfomycin resistance. As bacterial killing by some antibiotics is known to be attenuated at high compared to low initial inocula, bacterial killing against three strains was further examined at an initial inoculum of ~10^8 cfu/mL.

Prior to each experiment isolates were subcultured on nutrient agar (Media Preparation Unit) and incubated at 35ºC overnight. One colony was then randomly selected and grown overnight in 10 mL CAMHB at 37ºC from which the early-log-phase culture was obtained. Fosfomycin was then added to 20 mL of log-phase broth culture (CAMHB; containing 23.0 mg Ca^{2+}/L, 12.2 mg Mg^{2+}/L, and 25 mg/L G6P) to yield concentrations of 1, 4, 16, 32, 64, 128, 256, 512 and 1024 mg/L (~10^6 cfu/mL inoculum) and 4, 32, 128, and 512 mg/L (~10^8 cfu/mL inoculum). Each 20-mL culture was placed in a sterile 50-mL polypropylene tube (Thermofisher, Melbourne, Australia) and incubated in a shaking water bath at 37ºC for 24h.

As bacterial killing and the emergence of resistance may be affected by fluctuating as opposed to static drug concentrations, [291,292] antimicrobial activity and the emergence of resistance was additionally examined against the reference strain using a one-compartment pharmacokinetic/pharmacodynamic (PK/PD) *in vitro* model with an initial inoculum of ~10^6

cfu/mL. PAPs were conducted at baseline (0 h) and 24 h as per static time-kill studies. The chosen flow rate simulated a fosfomycin elimination half-life ($t_{½}$) of 4.0 h, which approximates the average half-life in a critically ill patient.[135,200] The flow rate was calibrated prior to and monitored throughout the experiment to ensure the system was performing optimally. This model has been described in detail previously.[293] In brief, a 1.0-mL aliquot of early-log-phase culture was inoculated into each compartment at the commencement of the experiment to yield ~10^6 cfu/mL. Shortly after, fosfomycin was injected into each treatment compartment (CAMHB; containing 23.0 mg Ca^{2+}/liter, 12.2 mg Mg^{2+}/liter, and 25 mg/L G6P) to achieve concentrations of 16, 63, 125, 250, 500 or 1000 mg/L.

For both static and dynamic models, serial samples (700 μL) were collected aseptically for viable counting at 0, 3, 5, 8 and 24 h. Given the high concentrations of antibiotic employed, antibiotic carryover was minimized by twice centrifuging samples at 10,000 rpm for 5 min immediately after sampling, with the supernatant discarded and bacteria resuspended to 700 μL with pre-warmed saline (37ºC) each time. Antibiotic carryover was further minimized by serial dilution, with 50 μL of bacterial cell suspension spirally plated onto nutrient agar (Media Preparation Unit) supplemented with G6P. Colonies were counted following 24 h of incubation (48 h for plates with small colonies) as described previously for PAPs.

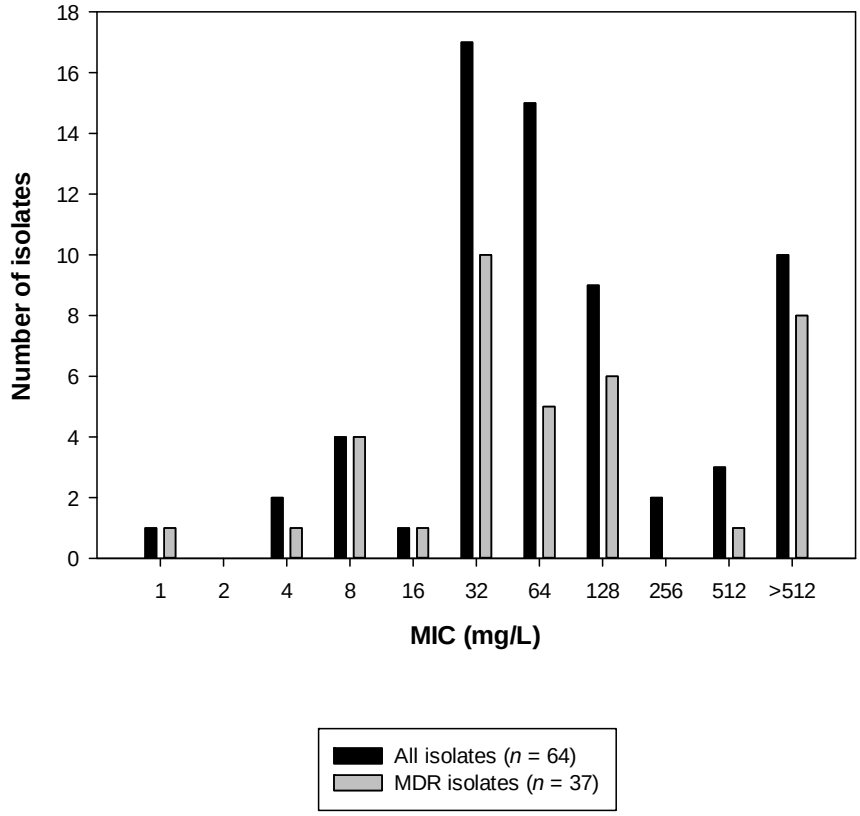
Results

MICs, PAPs and PAE

The MICs to fosfomycin and the MIC distribution are shown in Table 2.1 and Figure 2.1, respectively. Thirty seven of 64 (58%) clinical isolates were MDR. For all clinical isolates (MDR and non-MDR), 39 of 64 (61%) were susceptible to fosfomycin (MIC range, 1 – 64 mg/L); the corresponding values for strains taken from patients with cystic fibrosis and critically ill patients

were 36 of 59 (61%; MIC range, 1 – 64 mg/L) and 3 of 5 (60%; MIC range, 32 – 64 mg/L), respectively. When only MDR isolates were considered, 21 of 37 (57%) were susceptible (MIC range, 1 – 64 mg/L). MIC_{50} and MIC_{90} values across all isolates were 64 and >512 mg/L; this result was unchanged when only the MDR isolates were considered.

Figure 2.1. MIC distribution to fosfomycin of sixty-four clinical isolates of P. aeruginosa from Australia.



PAPs of ATCC 27853 and the fourteen clinical isolates at a high inoculum (~$10^{8.5}$ cfu/mL) are shown in Figure 2.2. Resistant subpopulations (i.e., subpopulations able to grow in the presence of >64 mg/L fosfomycin) were present in all strains examined in the PAPs despite an MIC range for these isolates of 1 – 64 mg/L. The proportion of bacterial colonies on plates containing

fosfomycin at 128 mg/L ranged from 1.10×10^{-6} – 3.69×10^{-4}, with a similar proportion of subpopulations growing in the presence of 256 mg/L. PAE durations for each isolate are shown in Table 2.2. The calculated PAE ranged from 0.3 – 5.5 h. The longest PAE was observed with strain CW 7 (2.5 – 5.5 h) and the shortest with strain CR 1005 (0.4 – 1.1 h) (Table 2.2). The duration of the PAE increased with increasing fosfomycin concentration.

Figure 2.2. Baseline PAPs of fourteen clinical isolates and ATCC 27853. All strains were considered susceptible based on MIC measurements (MICs ≤64 mg/L). The y-axis starts from the limit of detection.

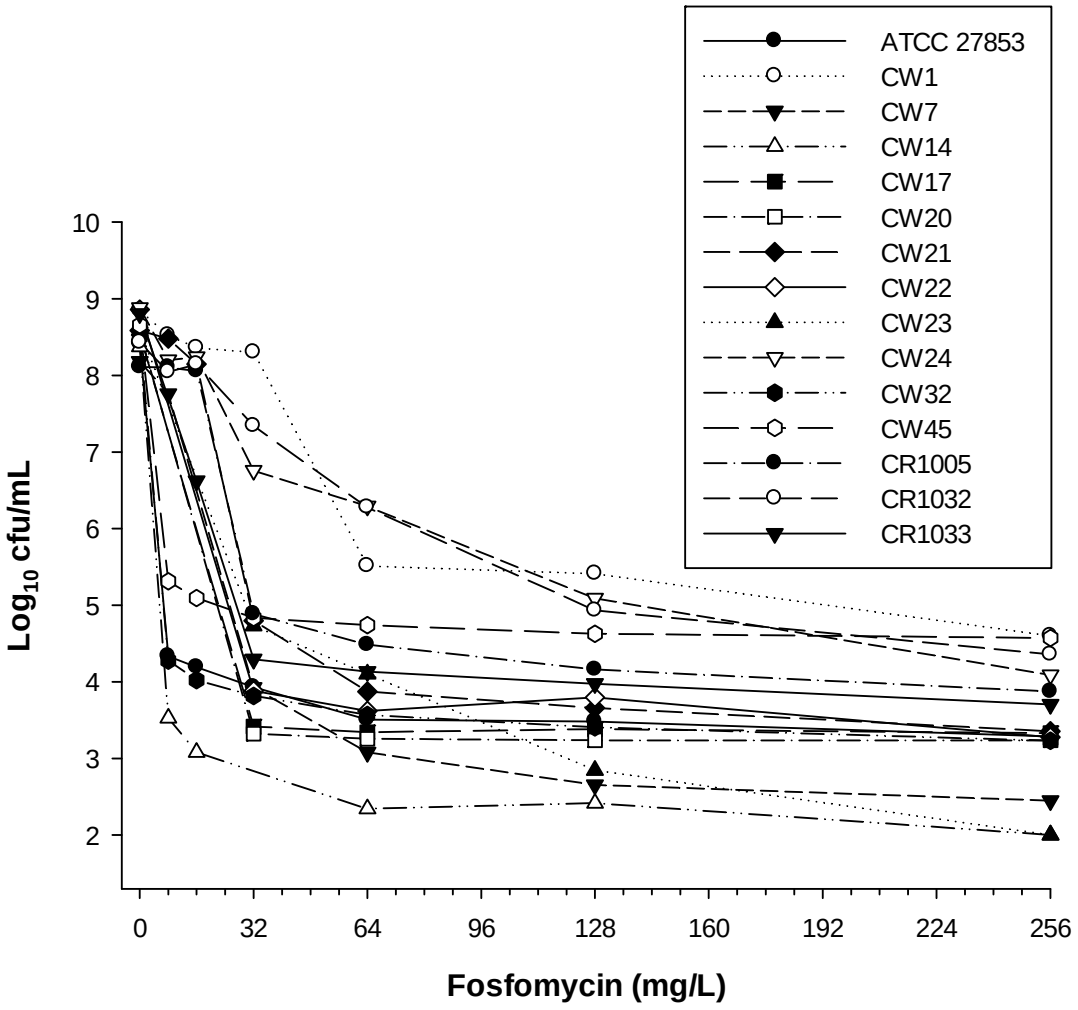


Table 2.2. PAE of fosfomycin for two clinical isolates and reference strain of P. aeruginosa

Strain	MIC (mg/L)	Fosfomycin concentration (mg/L)	PAE (h)
ATCC 27853	8	4	0.3
		32	1
		256	2.4
CW 7	16	4	2.5
		32	3.7
		256	5.5
CR 1005	32	4	0.4
		32	0.6
		256	1.1

Time-kill kinetics

The time course profiles of bacterial numbers achieved with ATCC 27853 and the representative clinical isolate CR 1005 for the static time-kill studies at both inocula, including the PAPs at the lower inoculum, are shown in Figure 2.3. Data for the remaining isolates is shown in the supplemental material (Figure S1). The time course profile and PAPs from the PK/PD *in vitro* model is shown in Figure 2.4. For static and dynamic experiments at ~10^6 cfu/mL inoculum, the majority of fosfomycin concentrations produced gradual killing of

bacteria within the first 3 – 5 h, with regrowth thereafter such that at 24 h growth had returned to, or was close to, control values. For two isolates (ATCC 27853 and CW 17) regrowth remained suppressed at 24 h with the highest fosfomycin concentrations (≥256 mg/L) in the static time-kill experiments. The maximum killing produced against any isolate at any fosfomycin concentration was ~3-$\log_{10}$ cfu/mL. This threshold was only just exceeded in the reference strain with two concentrations at 8 h (256 and 1024 mg/L, each producing 3.1 $\log_{10}$-killing; static time kills only) and with the 1024 mg/L regimen at 24 h (3.2 $\log_{10}$-killing). The rate of initial killing generally increased with increasing concentration up to ~64 – 256 mg/L (equivalent to ~4 – 8× MIC for each isolate) whereupon higher concentrations did not appear to produce faster or more extensive killing.

Figure 2.3. (Left panels) Time-kill curves for fosfomycin at an inoculum of ~10^6 cfu/mL. (Middle panels) PAPs at baseline (0 h) and after 24 h exposure to fosfomycin at an inoculum of ~10^6 cfu/mL. (Right panels) Time-kill curves for fosfomycin at an inoculum of ~10^8 cfu/mL. (A) ATCC 27853 (reference strain, MIC of 8 mg/L; non-MDR); (B) CR 1005 (MIC of 32 mg/L; MDR). The *y*-axis starts from the limit of detection.

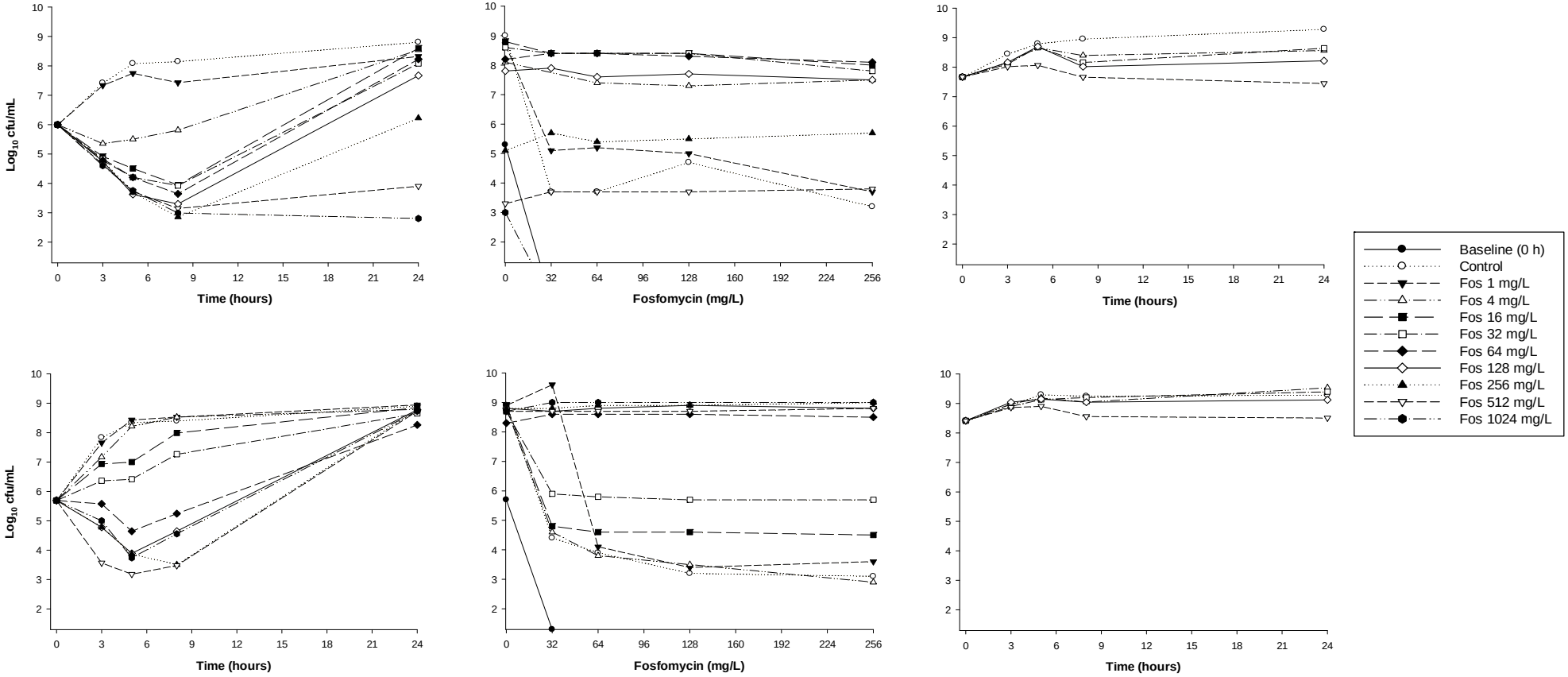
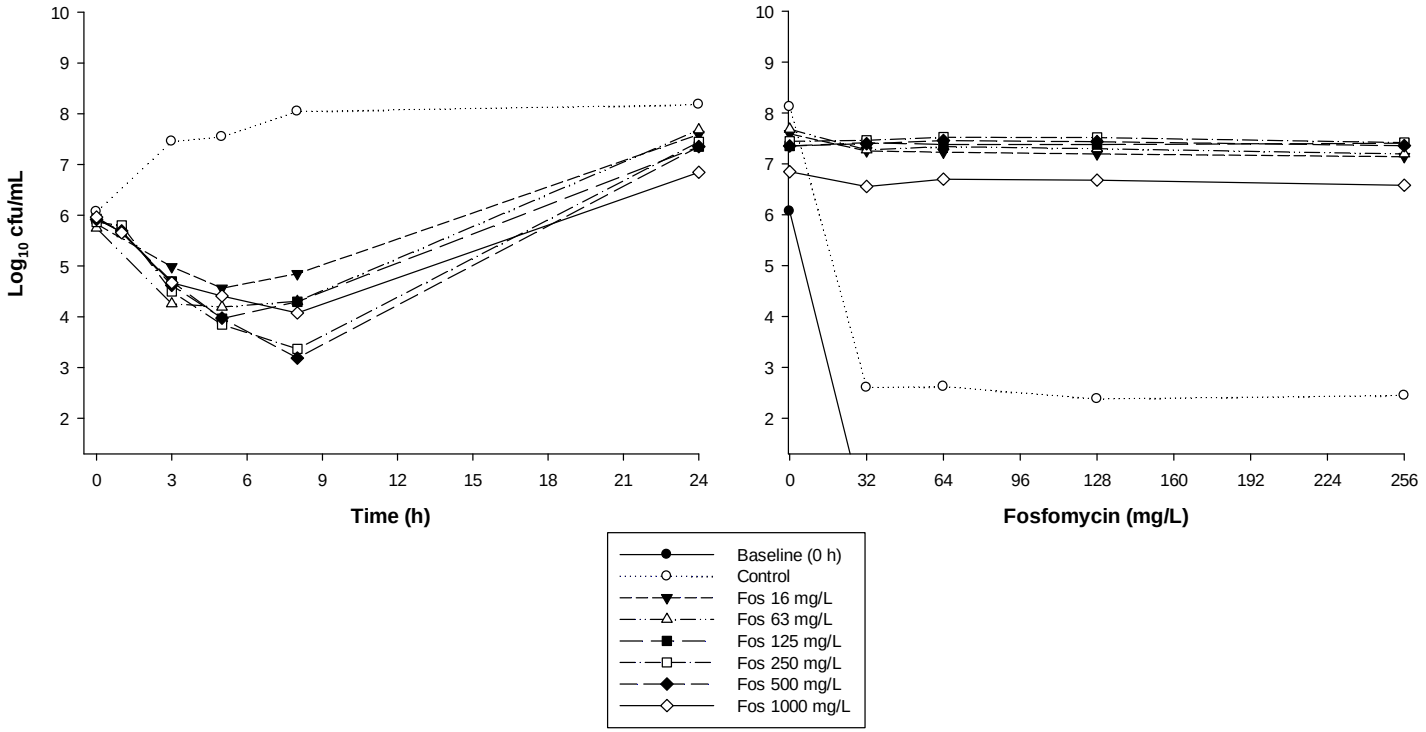


Figure 2.4. Reference strain ATCC 27853 (MIC to fosfomycin, 8 mg/L). (Left) Time-kill curves with various concentrations of fosfomycin at a starting inoculum of ~10^6 cfu/mL in a one-compartment *in vitro* pharmacokinetic/pharmacodynamic (PK/PD) model. (Right) PAPs at baseline (0 h) and after 24 h of exposure to fosfomycin. Fosfomycin was administered at the commencement of the experiment and a $t_{½}$ of 4.0 h was simulated. The *y*-axis starts from the limit of detection.



At the higher inoculum (~10^8 cfu/mL) in the static time-kill studies, bacterial killing was virtually eliminated even at the highest concentration (512 mg/L) for ATCC 27853 and CR 1005 with growth essentially no different to that of the control (Figure 2.3, right-hand panels); for isolate CW17 only a small amount of killing (<2 $\log_{10}$ cfu/mL) was observed at concentrations of 128 and 512 mg/L (Figure S2).

Emergence of fosfomycin resistance

No fosfomycin-resistant colonies were detected in the PAPs at baseline (0h; inoculum, ~10^6 cfu/mL) in the time-kill studies (Figure 2.3, middle panels; Figure 2.4, right-hand panel). For growth controls (ATCC 27853 [static and dynamic *in vitro* models] and CR 1005 [static model]), PAPs following 24 h incubation indicated the presence of resistant subpopulations. At this time, regrowth to ~$10^{8\text{-}8.5}$ cfu/mL had occurred. The proportion of resistant colonies (i.e. able to grow in the presence of fosfomycin at 128 mg/L) at 24 h was 5.0×10^{-5} [static model] and 1.6×10^{-6} [PK/PD model] for ATCC 27853 and 2.0×10^{-6} [static model] for CR 1005. These proportions were similar to those observed at baseline when tested at a higher inoculum (~$10^{8.5}$ cfu/mL) in the PAP studies examining heteroresistance.

At the lower static drug concentrations (1.0 mg/L for ATCC 27853 and 1, 4 and 16 mg/L for CR 1005), the proportion of resistant subpopulations at 24 h increased only slightly compared to that present in the controls. However, with higher concentrations in the static model and all concentrations (16 – 1000 mg/L) in the dynamic model essentially the entire population at 24 h was able to grow in the presence of fosfomycin at 256 mg/L (Figure 2.3, middle panels; Figure 2.4, right-hand panel). The only exception to this was fosfomycin at 1024 mg/L against the reference strain in the static model, where no colonies at all were detected at any concentration in the PAPs; at this time, regrowth was only ~3 $\log_{10}$ cfu/mL.

Discussion

Due to the growing threat from bacterial 'superbugs' there has been renewed interest in fosfomycin, an old antibiotic never subjected to contemporary drug development procedures. Prior to the present study, no systematic investigations on the *in vitro* pharmacodynamics of fosfomycin against any organism have been undertaken. The current study examined the pharmacodynamic properties of fosfomycin against clinical isolates of *P. aeruginosa*, including MDR isolates.

The susceptibility of *P. aeruginosa* to fosfomycin according to MIC results varies substantially in the literature and it has been suggested that antipseudomonal activity may be population dependent.[111] Such a situation thus requires familiarity with local susceptibility patterns for optimal use. Our study is the first to examine the susceptibility of *P. aeruginosa* isolates to fosfomycin within an Australian context. We found that 37 of 64 (58%) clinical isolates were considered susceptible to fosfomycin based upon the chosen breakpoints; when only the MDR isolates were considered, 21 of 37 (57%) isolates were susceptible. These results show that although a majority of the Australian isolates tested (including MDR strains) were fosfomycin-susceptible, a significant subset were resistant. Given the majority of isolates were from patients with CF treated at a single hospital, however, wider testing is required in order to establish susceptibility more generally in the Australian setting.

It is difficult to compare the rates of susceptibility of *P. aeruginosa* to fosfomycin observed in the present study to previous studies given the various testing methods employed (disk diffusion, agar dilution, broth microdilution and Etest) and a lack of universally accepted interpretive MIC breakpoints. Additionally, the proportion of MDR stains is often not specified. In a systematic review of fosfomycin conducted by Falagas *et al*.,[51] 7 of 19 studies found ≥90% of MDR *P. aeruginosa* isolates were susceptible to fosfomycin with an additional

4 studies reporting susceptibility rates between 50 – 90%. However, when the results of all studies were pooled the overall susceptibility rate was 30.2%. This substantially lower result was due to the great majority of the isolates (1348 of 1693) deriving from a single, older study which reported a fosfomycin susceptibility of only 20.9%;[294] that study was conducted in France where fosfomycin has been used routinely in clinical practice for the treatment of systemic infections. Many of the studies included were undertaken more than a decade ago. More recent studies have indicated overall susceptibility rates of between 31– 85%, with the majority not specifying whether isolates are MDR or non-MDR.[295-302] However, Perdigão-Neto *et al*.[295] reported that only 1 of 15 (7%) MDR isolates from Brazil was fosfomycin-susceptible, whereas a study by Maraki *et al*.[303] in Greece reported 8 of 9 (89%) MDR strains were susceptible. Of those studies, which have included at least 100 isolates of *P. aeruginosa* (MDR or non-MDR not specified), susceptibility ranged from 67 – 82%.[298,299,301] Such varying results clearly highlight the need for more uniformity of testing methods, as well as information on the pharmacodynamics of fosfomycin against *P. aeruginosa* so that organizations such as EUCAST will be able to establish effective breakpoint criteria. They also reinforce the need to determine local susceptibility patterns.

While the MIC remains the major parameter used to quantify the activity of an antimicrobial against a particular pathogen it provides no information about the activity of higher drug concentrations or persistent antimicrobial effects following drug exposure (i.e. the PAE). The effect of increasing concentrations on antimicrobial activity and the duration of persistent effects provide a much better description of the time course of antimicrobial activity than is provided by the MIC and are important considerations when designing dosage regimens. There is confusion in the literature, however, as to whether fosfomycin displays time- or concentration-dependent bactericidal activity. Time-dependent activity is characterized by saturation of the rate of killing with increasing concentrations, with higher concentrations not

producing faster or more extensive killing than lower concentrations.[194] Concentration-dependent activity is characterized by a greater rate and extent of killing with higher concentrations over a wide concentration range. In this regard it has recently been suggested that the type of activity displayed by fosfomycin may be organism dependent.[33] In the present study with both reference and clinical strains saturation of the rate of killing with increasing fosfomycin concentrations was observed. This indicates that bacterial killing of *P. aeruginosa* by fosfomycin is time-dependent (Figures 3, 4 and S1). Only one study has previously reported time-dependent bacterial killing of fosfomycin against *P. aeruginosa* and that was against a single reference strain (ATCC 27853) at a relatively low inoculum (~5.5× 10^6 cfu/mL).[79] We have confirmed this result with a larger number of isolates (including both MDR and non-MDR isolates) and with both static and dynamic concentrations. Time-dependent killing by fosfomycin has also been reported against *Staphylococcus aureus*.[79,200,205] In contrast, Mazzei *et al*.[199] reported concentration-dependent activity against *Escherichia coli* and *Proteus mirabilis*. However, in that study higher concentrations did not result in faster or more extensive bacterial killing than did lower concentrations and the data would appear to show time-dependent, not concentration-dependent, killing. Interestingly, a number of other papers are often cited in the literature in support of concentration-[207] or time-dependent killing[135,231] against various organisms. However, these studies did not examine concentration- or time-dependent activity and the authors made no claims to this effect. Irrespective of the type of activity against these other organisms, our data clearly indicates time-dependent activity against *P. aeruginosa*.

An interesting finding of our study is the extent of bacterial killing observed. Bactericidal activity for fosfomycin is consistently reported against a range of bacterial species. Previously published data for *P. aeruginosa*, though extremely limited, is more suggestive of bacteriostatic activity.[79,95,304] We observed primarily bacteriostatic activity. At an initial

inoculum of ~10^6 cfu/mL, a 3-$\log_{10}$ cfu/mL reduction in viable bacteria (i.e. bactericidal activity[305]) was only barely achieved against the reference strain in the static time-kill studies and only with two concentrations (3.1 $\log_{10}$-killing with 256 and 1024 mg/L at 8 h and 3.2 $\log_{10}$-killing with 1024 mg/L at 24 h); clinically, the maximum achievable concentrations of fosfomycin attained in plasma following intravenous administration in critically ill patients are in the range of 400 – 600 mg/L.[33] Regrowth occurred rapidly following initial killing, driven by amplification of a fosfomycin-resistant subpopulation (discussed below). We have also shown for the first time a pronounced inoculum effect for fosfomycin, with bacterial killing essentially eliminated at the high inoculum (~10^8 cfu/mL; Figure 2.3, right-hand panels). We included this second, higher inoculum as bacterial killing by some antibiotics is known to be attenuated at high compared to low initial inocula, including against *P. aeruginosa*.[209-213] Fosfomycin produced only a moderate PAE at higher concentrations with isolate CW 7 having a longer PAE than either the reference strain or CR 1005 (Table 2.2). Only MacLeod *et al*.[79] has previously investigated the PAE of fosfomycin against *P. aeruginosa*. In that study a PAE of 1 h was reported for the same reference strain (ATCC 27853) with a fosfomycin concentration of 8 mg/L, which is similar to the result reported here.

As noted above, regrowth occurred rapidly at the lower inoculum following initial modest bacterial killing even with concentrations well above those which are clinically achievable following intravenous therapy. This regrowth may be explained, at least in part, by the presence of pre-existing fosfomycin-resistant subpopulations as observed in the PAPs (Figure 2.2). Although no resistant colonies were observed at baseline in the time-kill studies, this is likely due to the lower initial inoculum thus greatly reducing the ability to detect such colonies. The presence and/or amplification of resistant subpopulations in isolates otherwise considered susceptible based on MIC measurements (i.e., heteroresistance) has not previously been reported for fosfomycin in any bacterial species. In this study, all isolates tested in the

PAPs at the high inoculum (~$10^{8.5}$ cfu/mL) contained a small but substantial proportion of resistant subpopulations despite MICs considered susceptible (Figure 2.2 and Table 2.1). These resistant subpopulations were able to grow even in the presence of 256 mg/L fosfomycin. Following 24 h exposure to both static and dynamic fosfomycin concentrations in the time-kill studies virtually all subpopulations grew in the presence of 256 mg/L fosfomycin. This suggests amplification of pre-existent highly-fosfomycin-resistant subpopulations within heteroresistant isolates is contributing to regrowth. Such a situation is analogous to that of another old class of antibiotics, the polymyxins, where regrowth of pre-existent polymyxin-resistant subpopulations is a known contributor to regrowth.[306] With polymyxins, the emergence in *P. aeruginosa* of colistin-resistance *in vitro* is known to occur more quickly when exposed to static, rather than dynamic, concentrations.[291,292] This, however, was not observed in the present study with the reference strain, where virtually the entire population grew in the presence of 256 mg/L fosfomycin in both static and dynamic models after 24 h of exposure. The rapid amplification of fosfomycin-resistant subpopulations observed indicates that fosfomycin monotherapy given intravenously is unlikely to be successful against *P. aeruginosa*. Given this situation, rational combination therapy with fosfomycin for systemic infections should be investigated to maximise bacterial killing and minimize the emergence of resistance.

In summary, the current study has demonstrated that while a majority of isolates (both MDR and non-MDR) of *P. aeruginosa* from two Australian hospitals were susceptible to fosfomycin, a substantial proportion were nevertheless resistant. Heteroresistance was observed in all fosfomycin-susceptible isolates examined. Bacterial killing of *P. aeruginosa* was bacteriostatic, time-dependent and markedly attenuated at higher inocula; PAE increased with increasing fosfomycin concentration. Importantly, substantial regrowth driven by the emergence of a highly fosfomycin-resistant subpopulation occurred even with fosfomycin

concentrations well above those that are clinically achievable in plasma following intravenous administration. These findings send a strong warning about the use of intravenous fosfomycin as monotherapy for systemic infection caused by *P. aeruginosa*. As resistance to other antimicrobials continues to increase, investigations of fosfomycin combination therapy to maximise bacterial killing and minimize the emergence of resistance are urgently warranted if fosfomycin is to be considered a viable therapeutic alternative against *P. aeruginosa*.

Acknowledgements

We are grateful to Dr Iain Abbott and A/Prof Denis Spelman (The Alfred Hospital, Melbourne) and Dr Hanna Sidjabat and Prof David Paterson (The Royal Brisbane & Women's Hospital, Brisbane) for providing the isolates studied. The assistance provided by Megan McGregor and Nada Millen (Monash University, Faculty of Pharmacy and Pharmaceutical Sciences, Parkville) in conducting experiments is gratefully acknowledged.

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Transparency declarations

None to declare.

Supplemental material

Figure S1. Time-kill curves for fosfomycin against three clinical isolates of *P. aeruginosa* at an inoculum of ~10^6 cfu/mL. (A) CW 7 (MIC of 16 mg/L; MDR); (B) CW 17 (MIC of 4 mg/L; MDR); CR 1033 (MIC of 32 mg/L; non-MDR). The *y*-axis starts from the limit of detection.

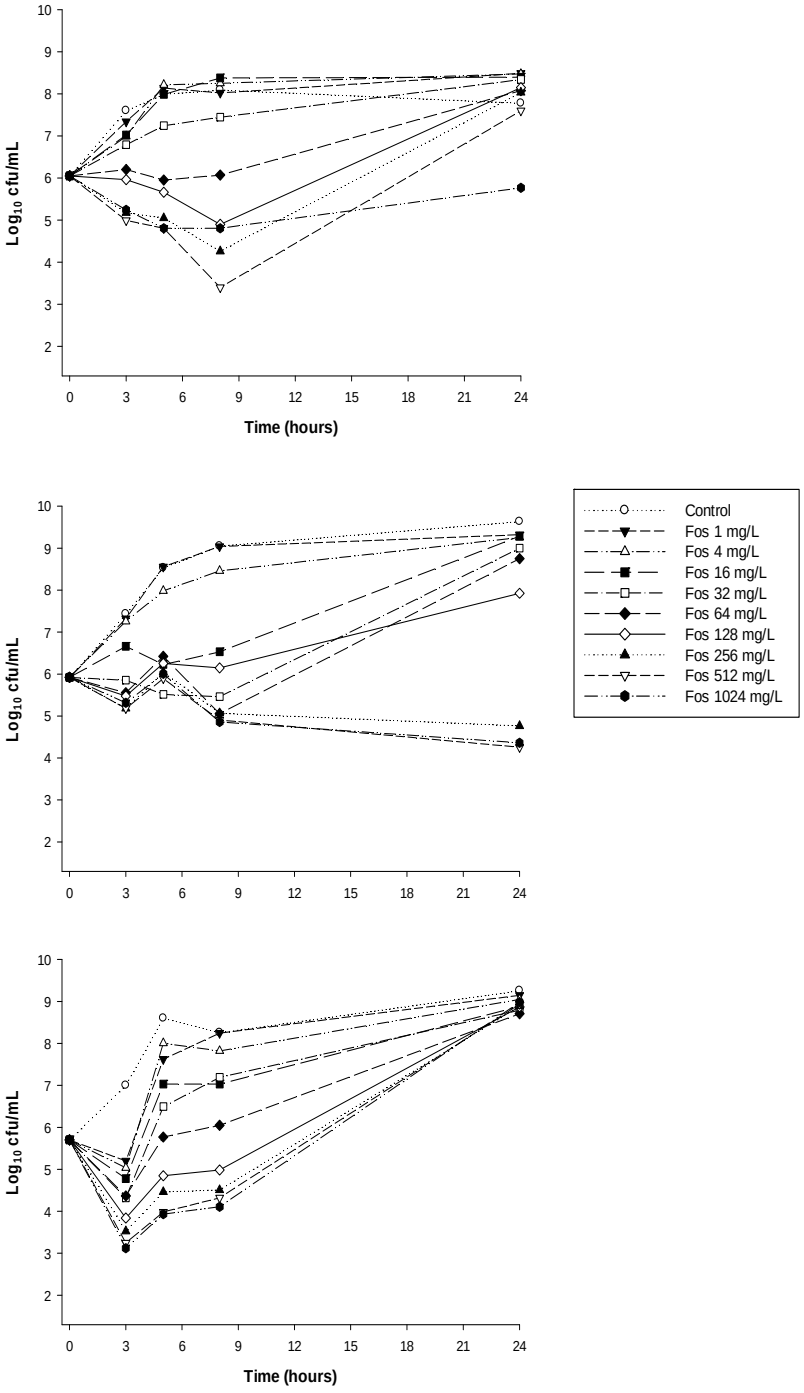
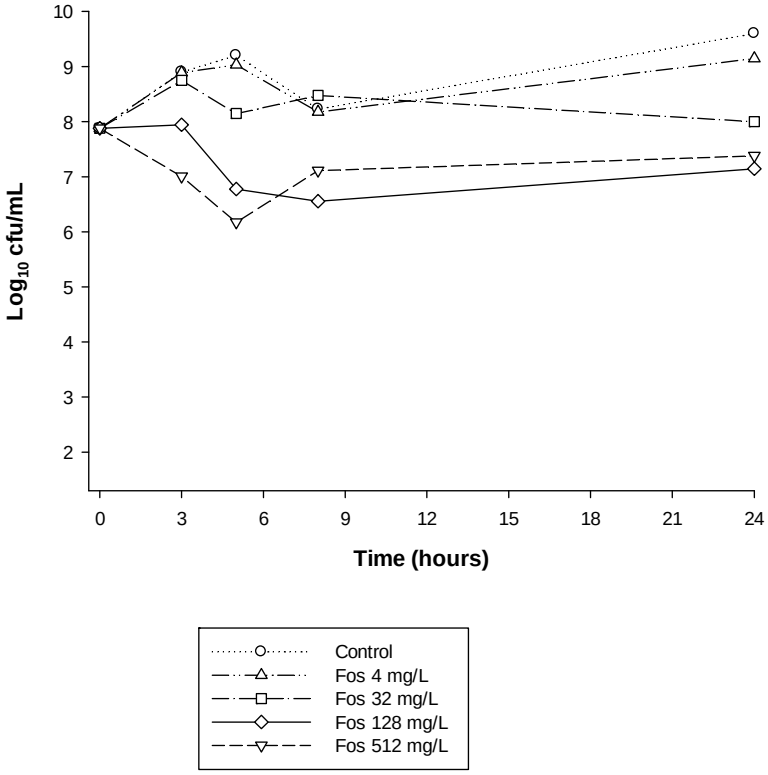


Figure S2. Time-kill curves for fosfomycin against clinical isolate CW 17 at an inoculum of ~10^8 cfu/mL. The *y*-axis starts from the limit of detection.



Declaration for Thesis Chapter Three

Declaration by candidate

In the case of Chapter Three, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
• design of the study; • all laboratory experiments; • data analysis and interpretation; • preparation of the initial draft of the manuscript and subsequent revisions; and • formulation of the conclusions and hypothesis arising from the results of the study.	80%

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution
Cornelia Landersdorfer	• review of manuscript drafts and revisions; and • formulation of conclusions and hypotheses arising from the results of the study.
Michelle McIntosh	• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation; • review of manuscript drafts and revisions; and • formulation of conclusions and hypotheses arising from the results of the study.
Anton Peleg	• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation; • review of manuscript drafts and revisions; and • formulation of conclusions and hypotheses arising from the results of the study.
Elizabeth Hirsch	• review of manuscript drafts and revisions; and • formulation of conclusions and hypotheses arising from the results of the study.
Carl Kirkpatrick	• supervision and advice regarding the concept and design of studies, laboratory experiments, data analysis and interpretation;

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

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature	[redacted]	**Date** 17/12/2015
Main Supervisor's Signature	[redacted]	**Date** 17/12/2015

Chapter Three

Clinically relevant concentrations of fosfomycin combined with polymyxin B, tobramycin, or ciprofloxacin enhance bacterial killing of *Pseudomonas aeruginosa* but do not suppress the emergence of fosfomycin resistance.

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Running title: Synergistic Killing of *P. aeruginosa* with Fosfomycin Combinations

Key words: fosfomycin, polymyxin B, tobramycin, ciprofloxacin, *Pseudomonas aeruginosa*, combination treatment, synergy, static time-kill

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Abstract

Objectives: Fosfomycin resistance occurs rapidly with monotherapy. This study systematically investigated bacterial killing and emergence of fosfomycin resistance with fosfomycin combinations against *P. aeruginosa*.

Methods: Four clinical isolates and a reference strain of *P. aeruginosa* were employed. Combinations of fosfomycin plus polymyxin B, tobramycin, or ciprofloxacin were examined over 24 h using time-kill studies (inocula ~10^6 cfu/mL) incorporating clinically relevant concentrations (fosfomycin, 30, 150 or 300 mg/L; polymyxin B, 0.5, 1 or 2 mg/L; tobramycin, 0.5, 1.5 or 4 mg/L; ciprofloxacin, 0.5, 1 or 2.5 mg/L). Microbiological response was examined by log changes and population analysis profiles.

Results: Against susceptible isolates, monotherapy produced varying degrees of initial killing followed by rapid regrowth. Fosfomycin plus polymyxin B or tobramycin produced greater initial killing (up to ~4-$\log_{10}$ cfu/mL) with many concentrations compared to monotherapy against fosfomycin-susceptible (Fos^S) isolates. With these combinations, synergy or additivity was observed in 54 (67%) and 49 (60%) of 81 cases (9 combinations across 3 isolates at 3 time points) for polymyxin B and tobramycin, respectively. Substantial improvements in killing were absent against fosfomycin-resistant (Fos^R) isolates. For fosfomycin-ciprofloxacin combinations, synergy or additivity was observed against Fos^R isolates in 33 of 54 (61%) cases (9 combinations across 2 isolates at 3 time points), while improvements in killing were largely absent against Fos^S isolates. No combination prevented emergence of fosfomycin resistance.

Conclusions: Against *P. aeruginosa*, fosfomycin in combination with polymyxin B or tobramycin (Fos^S isolates), or ciprofloxacin (Fos^R isolates), increased bacterial killing but did not suppress emergence of fosfomycin resistance.

Introduction

In recent times the world has faced a rapidly growing health crisis due to the emergence of multidrug-resistant (MDR) Gram-negative bacteria.[22,284] So serious has the problem become that unless new treatment options for these organisms are found society may soon be facing the dawn of the post-antibiotic era.[1] One Gram-negative bacterium of particular concern is the opportunistic pathogen *Pseudomonas aeruginosa*. A member of the ESKAPE pathogens – a group of pathogens known to 'escape' the bactericidal effects of many antibiotics through a wide range of drug resistance mechanisms – *P. aeruginosa* is capable of causing a broad range of infections in both community-dwelling and hospitalised patients, and is especially problematic in those with cystic fibrosis and the critically-ill.[307,308] Due to emerging resistance and few new antibiotics in the development pipeline, interest is growing in the redevelopment of older 'forgotten' antibiotics such as fosfomycin to help meet the need for alternative treatment options for MDR Gram-negative bacterial infections, including those caused by *P. aeruginosa*.[51,52,55,56,66-68]

Fosfomycin was discovered in the late 1960s and is currently indicated for the single-dose oral treatment of uncomplicated urinary tract infections (UTIs) caused by susceptible strains of *Escherichia coli* and *Enterococcus faecalis* in women.[75] It bears no structural relationship with other known antibiotics, represents its own antibiotic class, and shows no cross-resistance with other antimicrobials.[34] It exerts its antibacterial action by inhibiting the first cytoplasmic step in bacterial cell wall synthesis, disrupting the formation of the peptidoglycan precursor uridine diphosphate N-acetylmuramic acid (UDP-MurNAc).[149] The result is reduced peptidoglycan synthesis, reduced growth, and ultimately cell lysis.[149,309] It has a broad spectrum of activity against many Gram-negative and Gram-positive bacteria, including MDR strains, and is well tolerated with limited adverse effects at therapeutic

doses.[25,48,78] It is likely due to these favourable properties that clinicians have begun to re-examine the therapeutic potential of parenterally administered fosfomycin for the treatment of various infections other than UTIs caused by MDR Gram-negative and Gram-positive organisms.[25,32,51,55] However, resistance to fosfomycin emerges quickly when used as monotherapy, especially in *P. aeruginosa*, with no apparent fitness cost in this organism.[70,110,136,137] For this reason, fosfomycin is usually administered in combination with another class of antibiotic when used for treatment of systemic infections.[26,70] It has been suggested that fosfomycin combination therapy might protect against the emergence of resistance, although existing data are extremely limited.[253,310] Prior to the current investigation, no study has examined the emergence of fosfomycin resistance with combination therapy over time against any organism. The aim of this study was to systematically investigate the extent of *in vitro* bacterial killing and the emergence of fosfomycin resistance with clinically relevant concentrations of fosfomycin alone, and in combination, with polymyxin B, tobramycin or ciprofloxacin against *P. aeruginosa*. Such information is essential for the rational design of fosfomycin combination regimens.

Materials and methods

Bacterial strains and MIC measurements

Four clinical isolates of *P. aeruginosa* and reference strain ATCC 27853 (American Type Culture Collection, Manassas, VA, USA) were employed. All strains are described in detail in Table 3.1 and represented a mixture of susceptible and resistant strains. Three MDR clinical isolates (CW 2, CW 11, and CW 30; MDR defined as non-susceptibility to at least one antimicrobial agent in three or more antimicrobial categories[289]) were obtained from patients

with cystic fibrosis (CF) at the Alfred Hospital (Melbourne, Australia), and one non-MDR clinical isolate (CR 1033) was obtained from a critically ill patient at The Royal Brisbane and Women's Hospital (Brisbane, Australia). Fosfomycin (Waterstone Technology, Carmel, Indiana, USA; Lot 20131012) minimum inhibitory concentrations (MICs) were determined by agar dilution on Mueller-Hinton agar (Oxoid, Hampshire, England), whereas MICs of polymyxin B (Sigma-Aldrich, Castle Hill, Australia; Batch number BCBD1065V), tobramycin (Hospira Australia Pty Ltd, Mulgrave, Australia; Batch number A026741AA) and ciprofloxacin (Sigma-Aldrich, Castle Hill, Australia; Batch number 1298025) were determined in cation-adjusted Mueller-Hinton broth (CAMHB, Ca^{2+} at 23.0 mg/L, Mg^{2+} at 12.2 mg/L; Oxoid, Hampshire, UK) via broth microdilution.[311] MICs were determined in duplicate on separate days and all agar and CAMHB was supplemented with 25 mg/L glucose-6-phosphate (G6P; Sigma-Aldrich, Castle Hill, Australia; Lot SLBD7775V) in accordance with CLSI guidelines.[311] Susceptibility and resistance was defined as an MIC of ≤4 mg/L and >4 mg/L for tobramycin and ≤0.5 mg/L and >1 mg/L for ciprofloxacin as per the EUCAST guidelines.[106] Currently no breakpoints for fosfomycin or polymyxin B against *Pseudomon*as spp have been established. However, colistin and polymyxin B have comparable activity[47] and for the purposes of this study the colistin breakpoints of ≤4 mg/L for susceptibility and >4 mg/L for resistance were applied.[106] For fosfomycin, we applied modified CLSI breakpoints for the *Enterobacteriaceae* with an MIC ≤64 mg/L considered susceptible and >64 mg/L resistant.[311] All isolates susceptible to fosfomycin were heteroresistant (Fos^{HR}), defined as the presence within a fosfomycin-susceptible (Fos^{S}) isolate (i.e., MIC, ≤64 mg/L) of subpopulations able to grow on agar containing >64 mg/L fosfomycin.[136] Isolates were stored in tryptone soy broth (Oxoid, Hampshire, England) with 20% glycerol (Ajax Finechem, Seven Hills, NSW, Australia) in cryovials at -80°C and subcultured onto nutrient agar plates (Media Preparation Unit, University of Melbourne, Melbourne, VIC, Australia) before use.

Table 3.1: MICs for the P. aeruginosa isolates used in this study[a]

Isolate[b]	MIC (mg/L)[c]			
	Fos	PMB	Tob	Cip
ATCC 27853 n/m[d]	8	2	1	0.25
CW 2 muc[d,e]	64	>128	>128	16
CW 11 muc[e]	>512	0.5	8	0.125
CW 30 n/m[e]	>512	32	1	4
CR 1033 n/m[d]	32	2	1	0.25

[a] Fos, fosfomycin; PMB, polymyxin B; Tob, tobramycin; Cip, ciprofloxacin.

[b] muc, mucoid; n/m, non-mucoid.

[c] There are no established EUCAST or CLSI breakpoints for fosfomycin against *P. aeruginosa.* CLSI breakpoints for fosfomycin against the *Enterobacteriaceae* were adapted, with ≤64 mg/L considered susceptible and >64 mg/L resistant.[311] EUCAST breakpoints for tobramycin were ≤4 mg/L for susceptibility and >4 mg/L for resistance, and for ciprofloxacin ≤0.5 mg/L for susceptibility and >1 mg/L for resistance.[106] The EUCAST breakpoints for colistin of ≤4 mg/L for susceptibility and >4 mg/L for resistance were applied to polymyxin B.

[d] Fosfomycin heteroresistant. Heteroresistance to fosfomycin was defined as the presence within a fosfomycin-susceptible isolate (i.e., MIC, ≤64 mg/L) of subpopulations able to grow on agar containing >64 mg/L fosfomycin.[136]

[e] MDR, defined as resistant to at least one antimicrobial agent in three or more antimicrobial categories.[289]

Time-kill studies and the emergence of fosfomycin resistance

Bacterial killing and the emergence of fosfomycin resistance with combinations containing clinically relevant concentrations was examined using time-kill studies with each antibiotic alone or in combination (fosfomycin plus either polymyxin B, tobramycin, or ciprofloxacin) at an initial

inoculum of ~10^6 cfu/mL over 24 h. The concentrations employed were 30, 150 or 300 mg/L for fosfomycin,[112,200] 0.5, 1 or 2 mg/L for polymyxin B,[312] 0.5, 1.5 or 4 mg/L for tobramycin,[313,314] and 0.5, 1 or 2.5 mg/L for ciprofloxacin.[315]

Briefly, an early-log-phase culture was obtained and added to sterile 50 mL polypropylene tubes (Thermofisher, Melbourne, Australia) containing 20 mL of CAMHB (with 23.0 mg Ca^{2+}/L, 12.2 mg Mg^{2+}/L, and 25 mg/L G6P) plus either no drug (controls), monotherapy with each drug at one of three concentrations, or a combination of fosfomycin plus either polymyxin B, tobramycin, or ciprofloxacin at one of three concentrations; in total nine experiments (three concentrations of fosfomycin × three concentrations of the second antibiotic) for each fosfomycin combination were performed. Each tube was incubated in a shaking water bath at 37°C for 24 h. Serial samples were collected aseptically for viable counting at 0, 3, 5, 8 and 24 h. In order to evaluate the development of fosfomycin resistance, real-time population analysis profiles (PAPs) were additionally conducted on isolates CW 2 and CR 1033 using a limited number of concentrations of each drug at 0 and 24 h on nutrient agar containing fosfomycin at 32, 64, 128 and 256 mg/L. To minimize antibiotic carryover, samples were twice centrifuged at 10,000 rpm for 5 min and resuspended with pre-warmed saline (37°C), as well as serially diluted via spiral plating. Viable counting was performed as previously described.[136]

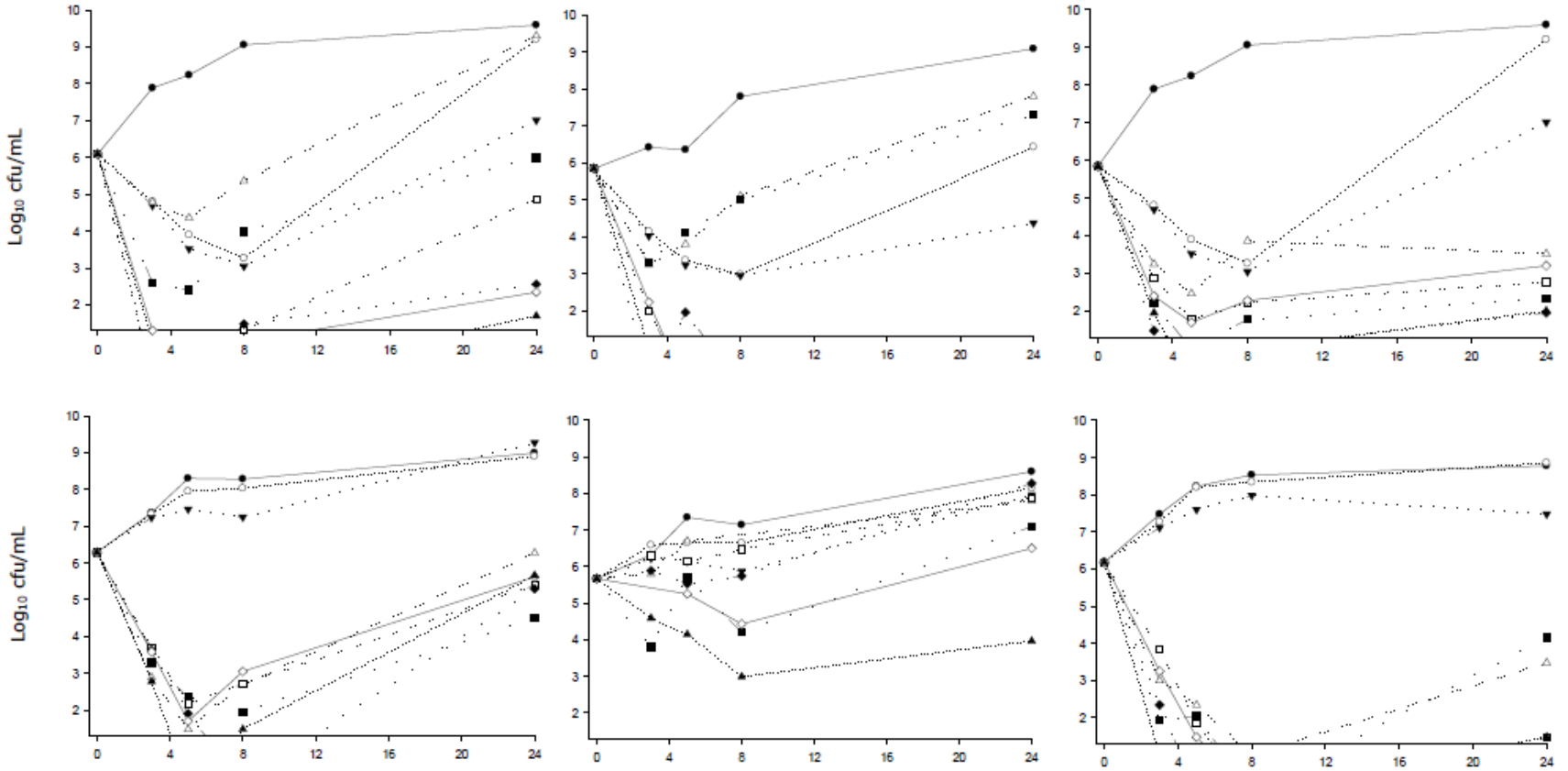
Pharmacodynamic (PD) analysis

Microbiological responses to mono- and combination- therapy were quantified using the log change method, which compares the change in $\log_{10}$ (cfu/mL) from 0 h (cfu_0) to time *t* (5, 8 or 24 h; cfu_t) according to the equation: log change = $\log_{10}(cfu_t) - \log_{10}(cfu_0)$. Synergy was defined as a reduction of $\geq$ 2-$\log_{10}$ cfu/mL for the combination compared to the most active monotherapy component at the specified time,[271] and additivity a reduction of 1 to <2-$\log_{10}$ cfu/mL for the combination. For both mono- and combination- therapy, activity was defined as a reduction of $\geq$ 1-$\log_{10}$ cfu/mL from the initial inoculum at 5, 8 or 24 h.

Results

For each antibiotic, representative time course profiles of bacterial numbers achieved with selected isolates and concentrations (monotherapy and combination therapy) are presented in Figures 3.1 and 3.2, with Figure 3.2 (right-hand panels) including baseline (0 h) and 24 h PAPs. The complete time-kill data for all isolates and concentrations are presented in colour in the Supplemental material. Log changes in viable-cell counts with mono- and combination- therapy are shown in Table 3.2 (fosfomycin and polymyxin B), Table 3.3 (fosfomycin and tobramycin), and Table 3.4 (fosfomycin and ciprofloxacin).

Figure 3.1. Representative time-kill curves with mid and high concentrations of fosfomycin (Fos) and polymyxin B (PMB) (left-hand panels), fosfomycin and tobramycin (Tob; middle panels), and fosfomycin and ciprofloxacin (Cip; right-hand panels) alone and in combination. Top row, ATCC 27853 (Fos^{HR}, PMB^{S}, Tob^{S}, Cip^{S}); Middle row, CW 11 (Fos^{R}, PMB^{S}, Tob^{R}, Cip^{S}); Bottom row, CW 2 (Fos^{HR}, PMB^{R}, Tob^{R}, Cip^{R}). The *y* axis starts from the limit of detection. All isolates and antibiotic concentrations are presented in color in the Supplementary material.



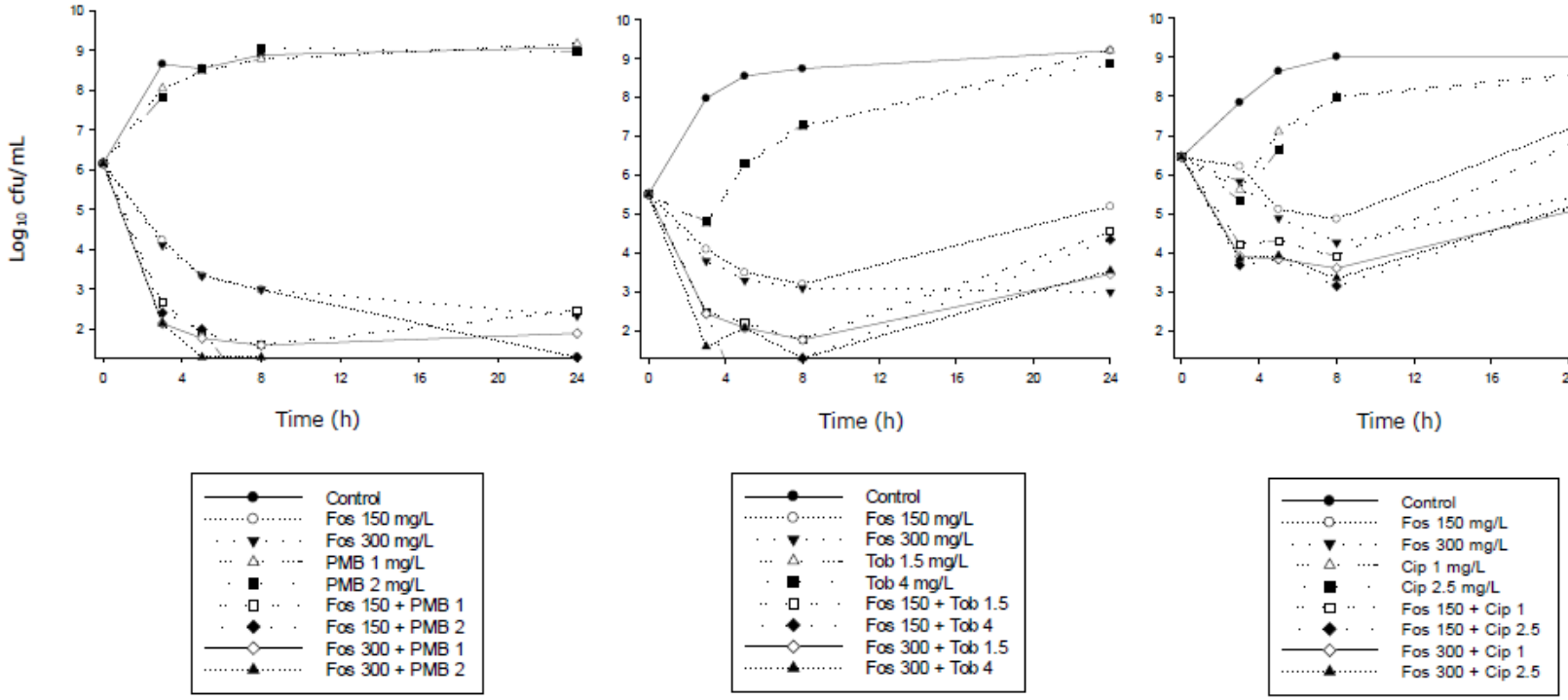

Log10 cfu/mL
Time (h)
Time (h)
Time (h)
Control
Fos 150 mg/L
Fos 300 mg/L
PMB 1 mg/L
PMB 2 mg/L
Fos 150 + PMB 1
Fos 150 + PMB 2
Fos 300 + PMB 1
Fos 300 + PMB 2
Control
Fos 150 mg/L
Fos 300 mg/L
Tob 1.5 mg/L
Tob 4 mg/L
Fos 150 + Tob 1.5
Fos 150 + Tob 4
Fos 300 + Tob 1.5
Fos 300 + Tob 4
Control
Fos 150 mg/L
Fos 300 mg/L
Cip 1 mg/L
Cip 2.5 mg/L
Fos 150 + Cip 1
Fos 150 + Cip 2.5
Fos 300 + Cip 1
Fos 300 + Cip 2.5

Figure 3.2. (Left) Representative time-kill curves with mid and high concentrations of fosfomycin (Fos) and polymyxin B (PMB; top row), fosfomycin and tobramycin (Tob, middle row), and fosfomycin and ciprofloxacin (Cip; bottom row) alone and in combination against CR 1033. (Right) PAPs at baseline (0 h; inoculum of ~10^6 cfu/mL) and after 24 h exposure to fosfomycin monotherapy and fosfomycin/second drug combination therapy, or neither antibiotic (control). The *y* axis starts from the limit of detection. All antibiotic concentrations are presented in color in the Supplementary material.

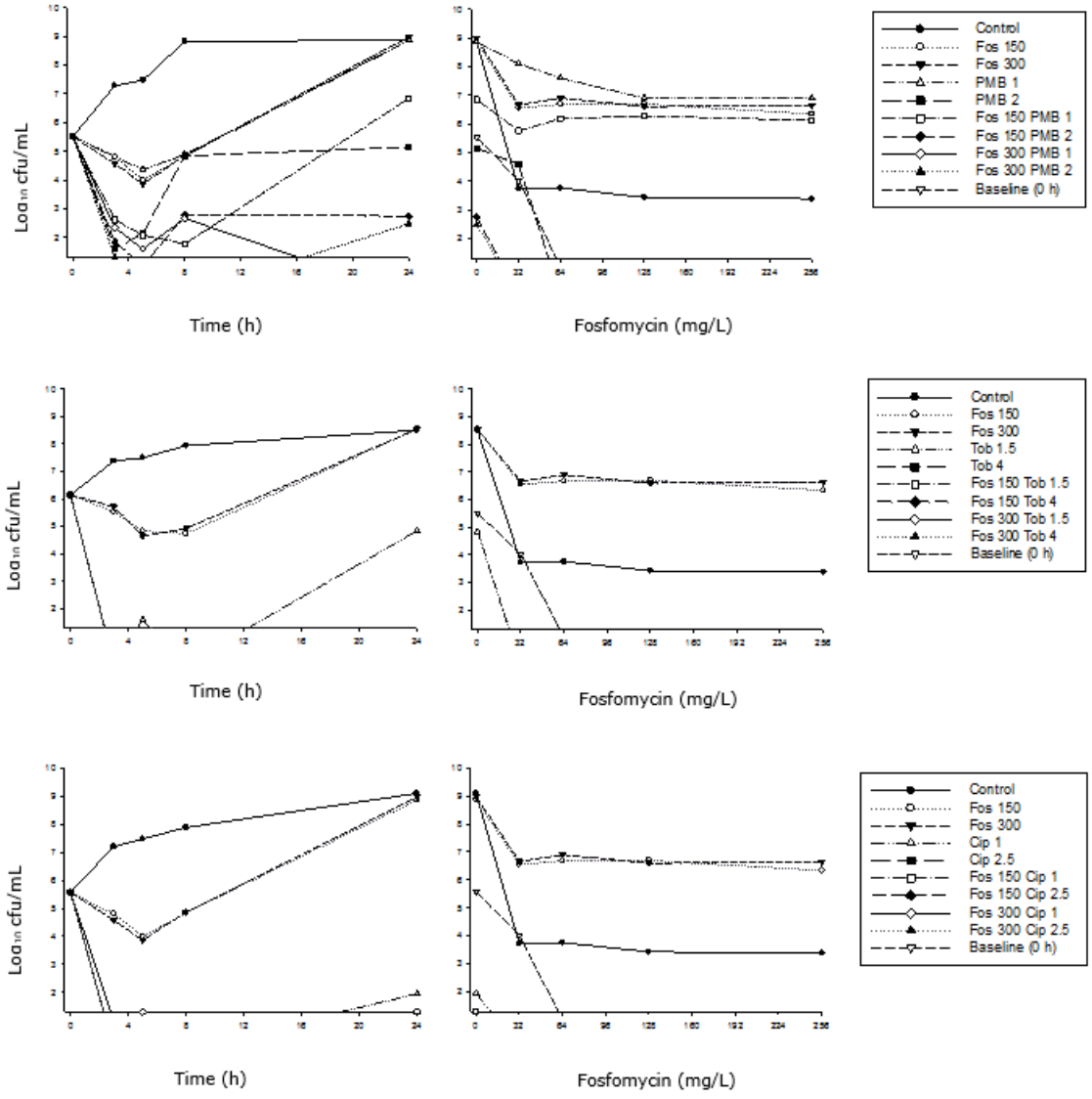


Table 3.2. Log changes at 5, 8 and 24 h with various clinically relevant concentrations of fosfomycin and/or polymyxin B against P. aeruginosa[a]

Isolate	Inoculum (cfu/mL)	Time (h)	Log change (= $\log_{10}(cfu_t)$ - $\log_{10}(cfu_0)$)														
			Fos 30 mg/L	Fos 150 mg/L	Fos 300 mg/L	PMB 0.5 mg/L	PMB 1 mg/L	PMB 2 mg/L	Fos 30 mg/L + PMB 0.5 mg/L	Fos 30 mg/L + PMB 1 mg/L	Fos 30 mg/L + PMB 2 mg/L	Fos 150 mg/L + PMB 0.5 mg/L	Fos 150 mg/L + PMB 1 mg/L	Fos 150 mg/L + PMB 2 mg/L	Fos 300 mg/L + PMB 0.5 mg/L	Fos 300 mg/L + PMB 1 mg/L	Fos 300 mg/L + PMB 2 mg/L
ATCC 27853	~10^6	5	-2.26	-2.19	-2.57	0.46	-1.73	-3.69	-2.91	-4.39	-6.09	-3.27	-6.09	-6.09	-3.47	-6.09	-6.09
		8	-1.99	-2.83	-3.05	2.37	-0.73	-2.11	-2.74	-3.46	-4.49	-3.86	-4.79	-4.61	-3.95	-5.09	-6.09
		24	3.35	3.11	0.92	3.51	3.21	-0.10	3.04	-2.21	-2.75	2.08	-1.22	-3.54	-3.38	-3.75	-4.39
CW 2	~10^6	5	1.54	-2.82	-2.82	2.47	2.30	2.37	0.41	0.56	0.50	-4.08	-4.26	-4.16	-4.38	-4.38	-4.86
		8	1.43	-3.17	-3.16	2.75	2.61	2.88	1.50	1.30	1.36	-6.16	-4.56	-6.16	-6.16	-4.56	-4.86
		24	2.61	-4.86	-3.82	2.99	3.00	2.81	2.74	2.50	2.57	-3.96	-3.69	-4.86	-3.91	-4.26	-6.16
CW 11	~10^6	5	1.61	1.66	1.18	-3.14	-4.81	-3.94	-3.39	-3.46	-3.02	-3.73	-4.11	-4.38	-3.59	-4.59	-6.29
		8	1.98	1.75	0.96	-3.82	-3.59	-4.33	-2.57	-3.56	-3.68	-3.71	-3.59	-6.29	-3.89	-3.24	-4.81
		24	2.68	2.62	2.99	1.53	-0.01	-1.79	0.41	-0.92	0.49	0.23	-0.89	-0.98	-1.02	-0.66	-0.62
CW 30	~10^6	5	-0.80	-0.96	-0.95	-0.02	-0.07	-0.54	-1.15	-1.20	-1.48	-2.64	-2.21	-2.87	-2.19	0.00	0.00
		8	0.00	-1.80	-2.30	1.66	1.77	1.66	-1.18	-1.43	-1.28	-2.28	-1.88	-2.17	-2.19	-1.67	-1.63
		24	1.70	1.50	1.60	2.58	2.87	2.99	1.82	0.90	1.87	0.44	0.20	0.08	0.12	0.68	0.29
CR 1033	~10^6	5	-1.65	-1.53	-1.65	1.94	-1.16	-3.37	-1.15	-3.31	-4.04	-1.88	-3.44	-4.52	-2.07	-3.92	-5.52
		8	-1.14	-0.67	-0.66	1.86	-0.63	-0.67	-0.97	-3.37	-1.77	-1.03	-3.74	-2.73	-0.70	-2.86	-5.52
		24	3.26	3.35	3.46	3.60	3.35	-0.37	3.50	0.60	-1.35	1.57	1.33	-2.77	1.75	-5.52	-3.04

[a] Fos, fosfomycin; PMB, polymyxin B. A grey background indicates activity (a reduction of ≥1 $\log_{10}$ cfu/mL below the initial inoculum); a green background indicates synergy (a ≥ 2 $\log_{10}$ decrease in the cfu/mL with the combination from that with its most active component); and a pink background indicates additivity (1- to < 2-$\log_{10}$ decrease in cfu/mL with the combination from that with its most active component).

Table 3.3. Log changes at 5, 8 and 24 h with various clinically relevant concentrations of fosfomycin and/or tobramycin against P. aeruginosa[a]

Isolate[b]	Inoculum (cfu/mL)	Time (h)	Log change (= $\log_{10}(cfu_t) - \log_{10}(cfu_0)$)														
			Fos 30 mg/L	Fos 150 mg/L	Fos 300 mg/L	Tob 0.5 mg/L	Tob 1.5 mg/L	Tob 4 mg/L	Fos 30 mg/L + Tob 0.5 mg/L	Fos 30 mg/L + Tob 1.5 mg/L	Fos 30 mg/L + Tob 4 mg/L	Fos 150 mg/L + Tob 0.5 mg/L	Fos 150 mg/L + Tob 1 .5 mg/L	Fos 150 mg/L + Tob 4 mg/L	Fos 300 mg/L + Tob 0.5 mg/L	Fos 300 mg/L + Tob 1.5 mg/L	Fos 300 mg/L + Tob 4 mg/L
ATCC 27853	~10^6	5	-1.87	-1.80	-2.18	-1.74	-2.04	-1.74	-2.88	-4.40	-5.70	-3.40	-5.70	-3.75	-4.00	-5.70	-5.70
		8	-1.61	-2.44	-2.66	-0.64	-0.74	-0.84	-2.36	-4.70	-5.70	-2.72	-4.70	-5.70	-3.17	-5.70	-5.70
		24	3.75	3.50	1.31	3.05	1.95	1.45	-4.61	-5.70	-5.70	-4.70	-5.70	-5.70	-4.40	-5.70	-4.70
CW 2	~10^6	5	-1.52	-2.02	-2.22	0.70	0.76	0.81	-0.91	-0.34	-0.58	-3.74	-3.31	-5.52	-3.52	-3.44	-3.44
		8	-0.82	-2.32	-2.42	1.74	1.72	1.79	-0.89	0.67	-0.45	-5.52	-3.74	-4.21	-3.91	-3.74	-4.21
		24	2.08	-0.32	-2.52	3.30	3.71	3.36	1.97	2.30	2.18	-0.90	-0.96	-1.17	-2.24	-2.05	-1.96
CW 11	~10^6	5	1.90	1.05	0.47	1.00	1.00	0.10	0.83	1.66	0.92	-0.31	0.52	-0.13	-1.93	-0.42	-1.59
		8	1.83	1.04	0.20	-0.40	1.20	-1.40	1.50	2.23	1.51	-0.10	0.85	0.09	-2.98	-1.24	-2.74
		24	2.83	2.54	2.29	2.50	2.10	1.50	2.91	2.75	2.73	2.84	2.25	2.62	-1.75	0.83	-1.77
CR 1033	~10^6	5	-0.92	-1.28	-1.38	-2.77	-4.71	-6.15	-3.16	-4.74	-6.22	-3.59	-6.29	-6.22	-3.99	-6.21	-6.09
		8	-0.92	-1.37	-1.09	-2.89	-6.31	-6.15	-4.48	-6.34	-6.22	-6.07	-6.29	-6.22	-6.19	-6.21	-6.09
		24	2.70	2.45	2.52	2.37	-1.47	-6.15	-3.50	-6.34	-6.22	-6.07	-6.29	-6.22	-6.19	-6.21	-6.09

[a] Fos, fosfomycin; Tob, Tobramycin. A grey background indicates activity (a reduction of ≥1 $\log_{10}$ cfu/mL below the initial inoculum); a green background indicates synergy (a ≥ 2 $\log_{10}$ decrease in the cfu/mL with the combination from that with its most active component); and a pink background indicates additivity (1- to < 2-$\log_{10}$ decrease in cfu/mL with the combination from that with its most active component).

[b] Fos plus Tob not tested against CW 30.

Table 3.4. Log changes at 5, 8 and 24 h with various clinically relevant concentrations of fosfomycin and/or ciprofloxacin against P. aeruginosa[a]

Isolate	Inoculum (cfu/mL)	Time (h)	Log change (= $\log_{10}(cfu_t) - \log_{10}(cfu_0)$)														
			Fos 30 mg/L	Fos 150 mg/L	Fos 300 mg/L	Cip 0.5 mg/L	Cip 1 mg/L	Cip 2.5 mg/L	Fos 30 mg/L + Cip 0.5 mg/L	Fos 30 mg/L + Cip 1 mg/L	Fos 30 mg/L + Cip 2.5 mg/L	Fos 150 mg/L + Cip 0.5 mg/L	Fos 150 mg/L + Cip 1 mg/L	Fos 150 mg/L + Cip 2.5 mg/L	Fos 300 mg/L + Cip 0.5 mg/L	Fos 300 mg/L + Cip 1 mg/L	Fos 300 mg/L + Cip 2.5 mg/L
ATCC 27853	~10^6	5	-2.02	-1.95	-2.33	-2.56	-3.38	-4.85	-3.22	-3.47	-5.85	-3.52	-4.07	-4.85	-3.43	-4.15	-5.85
		8	-1.75	-2.58	-2.81	-0.64	-1.99	-4.07	-1.13	-1.98	-4.55	-2.81	-3.62	-4.85	-3.03	-3.57	-4.85
		24	3.60	3.36	1.16	0.02	-2.33	-3.52	-2.68	-3.00	-3.43	-2.10	-3.08	-3.89	-2.94	-2.64	-3.85
CW 2	~10^6	5	0.37	-1.34	-1.57	0.66	0.66	0.19	0.66	0.83	-0.28	-2.20	-2.15	-2.62	-2.53	-2.62	-2.53
		8	0.93	-1.59	-2.20	1.75	1.55	1.52	0.57	0.41	0.73	-2.51	-2.55	-3.30	-2.72	-2.85	-3.11
		24	2.19	1.50	-0.67	2.33	2.30	2.15	1.71	1.45	2.11	1.04	1.26	-0.67	-0.43	-0.92	-0.67
CW 11	~10^6	5	1.76	2.02	1.42	-2.94	-3.84	-4.14	-3.20	-4.71	-6.18	-3.86	-4.34	-6.18	-4.07	-4.71	-6.18
		8	2.37	2.16	1.80	-2.69	-5.18	-6.18	-4.58	-6.18	-6.18	-6.18	-6.18	-6.18	-5.18	-6.18	-6.18
		24	2.52	2.68	1.30	2.06	-2.70	-2.02	-5.18	-4.71	-6.18	-6.18	-4.71	-6.18	-6.18	-6.18	-4.71
CW 30	~10^6	5	-0.67	-0.96	-0.95	-0.20	-0.50	-1.70	-0.12	-0.18	-0.91	-1.58	-1.25	-2.36	-1.64	-2.70	-3.20
		8	0.20	-1.76	-2.31	1.18	0.57	-1.16	-1.04	-1.39	-2.04	-2.61	-3.22	-3.92	-3.55	-3.24	-3.88
		24	1.88	1.55	1.57	2.27	1.94	-0.41	0.94	-0.12	-1.23	-1.14	-1.18	-5.70	-0.44	-0.47	-5.58
CR 1033	~10^6	5	-1.71	-1.59	-1.71	-3.43	-5.58	-5.58	-3.28	-5.58	-5.58	-3.05	-5.58	-5.58	-3.58	-4.28	-5.58
		8	-1.20	-0.73	-0.72	-3.43	-5.58	-5.58	-4.28	-5.58	-5.58	-4.28	-5.58	-5.58	-3.43	-4.58	-5.58
		24	3.20	3.29	3.40	1.10	-3.63	-5.58	-3.80	-3.47	-5.58	-3.58	-4.28	-5.58	-3.98	-4.58	-5.58

[a] Fos, fosfomycin; Cip, ciprofloxacin. A grey background indicates activity (a reduction of ≥1 log10 cfu/mL below the initial inoculum); a green background indicates synergy (a ≥ 2 log10 decrease in the cfu/mL with the combination from that with its most active component); and a pink background indicates additivity (1- to < 2-log10 decrease in cfu/mL with the combination from that with its most active component).

Monotherapy

Against isolates resistant to fosfomycin (Fos^R; CW 11, CW 30) or polymyxin B (PMB^R; CW 2, CW 30), monotherapy with the respective antibiotic at all concentrations produced little or no bacterial killing with growth paralleling that of the growth control (Figure 3.1 and S1). For isolates resistant to tobramycin (Tob^R; CW 2, CW 11) or ciprofloxacin (Cip^R; CW 2, CW 30), monotherapy with the respective antibiotic produced initial killing of <~1.5 log_{10} cfu/mL with subsequent rapid regrowth (Figure 3.1 and S1). Against susceptible isolates, monotherapy with each antibiotic at the concentrations used produced varying degrees of initial killing (within the first 3 h) followed by rapid regrowth in most cases (Figures 3.1 and 3.2, and S1). Initial killing was generally between ~1 to 3-log_{10} cfu/mL with fosfomycin, ~0.5 to 5-log_{10} cfu/mL with polymyxin B, ~2 to 6-log_{10} cfu/mL with tobramycin, and ~1 to 6-log_{10} cfu/mL with ciprofloxacin. At 24 h, for each of fosfomycin, polymyxin B and tobramycin, growth remained below the initial inoculum in only 2 of 9 cases (3 antibiotic concentrations across 3 isolates) against isolates susceptible to each drug; for ciprofloxacin, the equivalent value was 4 of 9 cases against susceptible isolates.

In the PAPs (isolates CR 1033 and CW 2; both Fos^{HR}), no Fos^R colonies were detected at baseline (0 h; inoculum ~10^6 cfu/mL) (Figure 3.2 and S1). Following 24 h of incubation and with growth reaching ~10^9 cfu/mL, the proportion of resistant colonies in the growth controls was 4.7×10^{-6} and 4.8×10^{-6} for CR 1033 and CW 2, respectively. With fosfomycin monotherapy against CR 1033, the proportion of resistant colonies at 24 h was similar with all concentrations (range: 6.0×10^{-3} – 9.1×10^{-3}). For CW 2, the proportion of resistant colonies increased with increasing fosfomycin concentration and was 6.9×10^{-5}, 1.1×10^{-2} and 1.7×10^{-1} with concentrations of 30, 150 and 300 mg/L, respectively.

Combination therapy

Fosfomycin plus polymyxin B. For the non-MDR Fos^S-PMB^S isolates (ATCC 27853 and CR 1033) the combination produced more rapid and extensive killing (up to ~4-log_{10} cfu/mL with many combinations) than with either antibiotic alone (Figures 3.1 and 3.2 and S1). In 6 of 18 combinations (9 combinations across 2 isolates), no viable colonies were detected within the first 5 hours. Synergy or additivity was observed in 39 of 54 (72%) cases (9 combinations across 2 isolates at 3 time points [5, 8, and 24 h]), especially with combinations containing polymyxin B at 1 or 2 mg/L, and occurred across the entire 24 h period (Table 3.2). At 24 h, regrowth was observed with all but one combination (fosfomycin 300mg/L plus polymyxin B 1 mg/L). However, in the majority of cases, and with all combinations containing polymyxin B at 2 mg/L, regrowth at 24 h was below both the initial inoculum and the 24 h count of the most effective monotherapy (polymyxin B at 2 mg/L). Bacterial killing was also enhanced for the MDR Fos^S-PMB^R isolate (CW 2) with the combination, although to a lesser extent. For this isolate the combination was additive or synergistic in 11 (41%) and 4 (15%) of 27 cases, respectively, with enhanced killing primarily limited to combinations containing fosfomycin at 150 or 300 mg/L across the initial 8 h of therapy. For CR 1033 and CW 2, PAPs at 24 h revealed that the proportion of Fos^R subpopulations was similar to that of equivalent fosfomycin monotherapy (Figure 3.2 and S1).

For the MDR Fos^R-PMB^S isolate (CW 11), synergy or additivity was only observed in 5 of 27 (19%) cases, with combination therapy generally mirroring that of the equivalent polymyxin B monotherapy. For the MDR Fos^R-PMB^R isolate (CW 30), synergy was not observed although all combinations containing fosfomycin 30 mg/L were additive at 8 h, and most combinations containing fosfomycin 150 or 300 mg/L were additive at 5 h.

Fosfomycin plus tobramycin. For the non-MDR Fos^S-Tob^S isolate (ATCC 27853), the combination at all concentrations produced substantially more rapid and extensive initial killing than with either antibiotic alone, with synergy (primarily) or additivity observed in 24 (89%) of 27 cases across 24 h (Figure 3.1 and Table 3.3). All combinations containing tobramycin concentrations of 1.5 or 4 mg/L resulted in no viable colonies being detected on multiple occasions. Enhanced bacterial killing was less pronounced across the first 8 h of treatment with the other isolate susceptible to both antibiotics (CR 1033; non-MDR). However, at 24 h all combinations containing tobramycin 0.5 or 1.5 mg/L were synergistic with ≥~5 $\log_{10}$ cfu/mL additional killing compared to the most effective monotherapy. The exception was the fosfomycin 30 mg/L plus tobramycin 0.5 mg/L combination, where no viable bacteria were detected at this time with combination therapy. With this isolate, tobramycin monotherapy at 4 mg/L resulted in bacterial eradication, precluding synergy.

For the MDR Fos^S-Tob^R isolate (CW 2), all combinations containing fosfomycin at 150 or 300 mg/L were additive or synergistic at both 5 and 8 h, however only one remained so at 24 h. The proportion of Fos^R subpopulations on the PAPs once again mirrored that of equivalent fosfomycin monotherapy (S1). For the MDR Fos^R-Tob^R isolate (CW 11), additivity or synergy was essentially restricted to combinations containing 300 mg/L fosfomycin. However, even with these combinations bacterial killing never exceeded 3-$\log_{10}$ cfu/mL at any time point.

Fosfomycin plus ciprofloxacin. For the non-MDR Fos^S-Cip^S isolates (ATCC 27853 and CR 1033), synergy or additivity was primarily observed with combinations containing ciprofloxacin at the lowest concentration (0.5 mg/L) and only at 24 h (Table 3.4). At this time, there was an additional ~5 $\log_{10}$ cfu/mL killing with all fosfomycin/ciprofloxacin 0.5 mg/L combinations (growth was below ~3.17 $\log_{10}$ cfu/mL) with no Fos^R colonies detected in the PAPs (Figure 3.2 and S1). Against the MDR, Fos^S-Cip^R isolate (CW 2), regrowth with all but one

combination (fosfomycin 150 mg/L plus ciprofloxacin 2.5 mg/L) approximated that of equivalent fosfomycin monotherapy. For this isolate, the proportion of Fos^R subpopulations in the PAPs at 24 h was similar to that observed with equivalent fosfomycin monotherapy (S1).

For the MDR Fos^R and Cip^S/Cip^R isolates (CW 11 and CW 30, respectively), bacterial killing was greatly enhanced with combination therapy with synergy or additivity observed in 19 (70%) and 14 (52%) of 27 cases (9 combinations across 3 time points), respectively. For the MDR Fos^R-Cip^S isolate (CW 11), all combinations were synergistic at 24 h with additional bacterial killing of up to ~8 log compared to the most effective monotherapy. At this time, viable colonies were detected with 6 of the 9 combinations. For the MDR Fos^R-Cip^R isolate (CW 30), enhanced bacterial killing was primarily restricted to combinations containing fosfomycin concentrations of 150 and 300 mg/L, especially when combined with ciprofloxacin at 2.5 mg/L. With these two combinations (fosfomycin 150 or 300 mg/L plus ciprofloxacin 2.5 mg/L), >5 $\log_{10}$ cfu/mL additional killing was achieved over equivalent monotherapy at 24 h, with regrowth at this time below the limit of quantification.

Discussion

With the increasing emergence of MDR Gram-negative bacteria there has been renewed interest in the use of 'forgotten' antibiotics such as fosfomycin. Indeed, there are increasing reports of fosfomycin being used in the clinic for treatment of infections due to MDR organisms, including *P. aeruginosa*.[39,61,62,148] However, concerns have been raised about the use of fosfomycin as monotherapy.[110,136] We recently showed that activity of fosfomycin against *P. aeruginosa* was bacteriostatic, even with concentrations well in excess (up to 1024 mg/L) of those which are usually achieved in plasma in patients following intravenous therapy.[136] Additionally, regrowth of Fos^{HR} isolates with monotherapy in that study was driven, at least in part, by amplification of pre-existing Fos^R subpopulations. Due to the failure of monotherapy to eradicate this organism and

the potential for the rapid emergence of fosfomycin resistance, fosfomycin combination therapy has been suggested as a way to both enhance bacterial killing and minimise the emergence of fosfomycin resistance.[38,253,310] However, in the clinic, combinations are often employed in the hope of improving the activity of agents when therapeutic options are limited. In such cases the choice of agents is often empirically driven and based on trial and error or personal experience. In this study we systematically investigated the effectiveness of fosfomycin alone and in combination with polymyxin B, tobramycin or ciprofloxacin against *P. aeruginosa*, including MDR, Fos^{HR} and Fos^{R} isolates. The second antibiotics were chosen due to their generally high activity against *P. aeruginosa* and previous studies (discussed below) indicating the potential utility of such combinations.[47,316-318]

The concentrations of fosfomycin and each second antibiotic were chosen to reflect low, mid and high average steady-state unbound plasma concentrations typically achieved in critically ill patients receiving standard doses of these antibiotics, and are thus clinically relevant.[112,200,312-315] Rapid and substantial regrowth of all isolates was observed with fosfomycin monotherapy, consistent with previous reports.[95,136,278,282] Recently, we demonstrated in *P. aeruginosa* that amplification of pre-existing Fos^{R} colonies in Fos^{HR} isolates is at least partly responsible for regrowth.[136] This was once again seen here in the PAPs, with substantial increases in the number of Fos^{R} colonies (*i.e.* growing in the presence of 128 mg/L fosfomycin) observed at 24 h with fosfomycin monotherapy compared to the controls. Although no Fos^{R} colonies were detected at baseline (0 h; inoculum ~10^{6} cfu/mL), in our previous report the proportion of resistant colonies for isolate CR 1033 prior to fosfomycin exposure was 1.5 x 10^{-5} using a higher initial inoculum (~$10^{8.5}$ cfu/mL). Thus, the absence of Fos^{R} colonies at baseline in the PAPs of the present study is likely due to the difficulties of detecting such colonies at the considerably lower inoculum used, rather than their absence. The possibility of such rapid amplification of resistant subpopulations

in isolates otherwise considered susceptible based on MIC measurements suggests care is required with fosfomycin monotherapy against *P. aeruginosa* regardless of susceptibility.

The addition to fosfomycin of polymyxin B or tobramycin generally resulted in substantial improvements in bacterial killing (up to ~4-$\log_{10}$ cfu/mL with many combinations and viable counts below the limit of detection) over 24 h compared with equivalent monotherapy for Fos^S isolates with the majority of combinations. This was particularly the case when the isolate was additionally susceptible to the second drug, although improvements in killing were also observed in isolates resistant to the second drug, especially with combinations containing fosfomycin at 150 and 300 mg/L. The benefits of these combinations on overall antibacterial activity against MDR Fos^R isolates were much less pronounced, irrespective of the susceptibility to the second drug. The situation with fosfomycin-ciprofloxacin combinations was somewhat the reverse, with improved bacterial killing of the MDR Fos^R isolates (including both Cip^S and Cip^R isolates) with the majority of combinations across 24 h, and virtually no improvement in effect on Fos^S isolates (with the exception of combinations containing ciprofloxacin at 0.5 mg/L at 24 h against the two Fos^S and Cip^S isolates). However, at the higher ciprofloxacin concentrations (1 and 2.5 mg/L), substantial bacterial killing occurred with ciprofloxacin monotherapy against two of the three Fos^S isolates, which may explain the lack of benefit observed with the combination. Nevertheless, even with these isolates, all combinations containing ciprofloxacin at 0.5 mg/L were synergistic at 24 h, with additional bacterial killing of ~5-log for clinical isolate CR 1033 at this time. This indicates substantial improvements in bacterial killing are possible with this combination even with each agent at lower concentrations.

A number of existing preclinical investigations of fosfomycin combination therapy with a variety of antibiotics, including those utilized in this investigation, have shown good synergistic effects against *P. aeruginosa* (including MDR isolates) despite many isolates being resistant to

the second drug.[49,79,95,266,267,276-278,280,282,319-321] However, many of these studies utilised the fractional inhibitory concentration (FIC) index or Etest methods to quantitatively define synergy, additivity, indifference or antagonism. Such methods are less discriminatory than time-kill methods, which measure the bactericidal activity of the combination and provide a picture of antimicrobial action over time. Remarkably few studies have employed time-kill methodology to assess pharmacodynamic interactions of fosfomycin combinations against *P. aeruginosa* and, until the present investigation, none of these have examined the effect of the combinations on the emergence of fosfomycin resistance.[95,278,282,319]

Only one study each has examined fosfomycin combined with a polymyxin or ciprofloxacin using time-kill methodology, and only two studies have examined fosfomycin plus tobramycin. Di *et al*.[95] examined fosfomycin-colistin combinations (each at 0.5× or 1× the MIC) against five carbapenem-resistant *P. aeruginosa* isolates (including two Fos^R isolates) of unknown MDR status. Combinations at both concentrations resulted in bacterial eradication in almost all cases, with no viable bacteria detected at 24 h; in contrast, regrowth with all monotherapies at this time was above ~10^7 log_{10} cfu/mL. Interestingly, such improvements in killing were achieved against Fos^S isolates with polymyxin concentrations comparable to the present study (range: 0.25 – 4 mg/L colistin) but with relatively low fosfomycin concentrations (range: 16 – 64 mg/L). Although we observed substantial improvements in killing with the fosfomycin-polymyxin B combination against Fos^S isolates, such improvements were not to the extent seen by Di *et al*. even with fosfomycin at 300 mg/L, and bacteria were eradicated in only one instance. We did not observe similar improvements against Fos^R isolates. Yamada *et al*.[280] observed enhanced bacterial killing (~2 – 4-log) over 10 h with a fosfomycin-ciprofloxacin combination (each at 0.5× MIC; fosfomycin concentrations ranged from 16 – 64 mg/L) against four clinical isolates of Cip^R *P. aeruginosa* (one strain Fos^R). We observed a similar improvement in killing at 8 h in only one

of two Cip^R isolates. For tobramycin, Díez-Aguilar *et al*.[278] recently reported a fosfomycin-tobramycin combination to have virtually no beneficial effect on bacterial killing of eight *P. aeruginosa* clinical isolates (all with a fosfomycin MIC of 64 mg/L; five Tob^S and three Tob^R). However in that study, the concentrations of fosfomycin and tobramycin employed were 90 mg/L and 10 mg/L, respectively, and tobramycin monotherapy rapidly eliminated viable bacteria against the susceptible isolates precluding any potential benefit with the combination. No benefit was observed with the combination against the three Tob^R isolates. In contrast, MacLeod *et al*.[282] examined fosfomycin (12.8 mg/L) plus tobramycin (3.2 mg/L) against *P. aeruginosa* reference strain ATCC 27853. The combination resulted in greatly enhanced early bacterial killing compared to equivalent monotherapy, with bacterial eradication by 6 h. In the present investigation, we have observed increased bacterial killing against isolates susceptible to both antibiotics as well as, at higher fosfomycin and/or tobramycin concentrations, Tob^R isolates.

To the best of our knowledge our study is the first to investigate the emergence of fosfomycin-resistance with combination therapy over time against any organism. Previously, a small number of studies have examined single-step resistance to fosfomycin and its combinations against *P. aeruginosa*.[70,79,137,282] MacLeod *et al*.[79] examined the development of resistance of *P. aeruginosa* to fosfomycin, tobramycin, and their combination after a single exposure (4× the MIC) to the antibiotics. Although the spontaneous mutation frequency (*i.e.* the number of colonies growing on antibiotic-containing agar plates divided by the number of colonies growing on antibiotic-free agar) resulting in resistance after a single exposure to the combination was less than the frequencies of fosfomycin or tobramycin, the differences were ≤ 2 $\log_{10}$ relative to tobramycin alone. However, in a subsequent study by the same group the mutation frequency of a fosfomycin-tobramycin combination at 16× the MIC was ≥ 3 $\log_{10}$ and 4 $\log_{10}$ lower than for fosfomycin and tobramycin alone, respectively.[282] Similar results were reported by McCaughey *et al*.[283] In similar

studies involving a reference strain of *P. aeruginosa* and its hypermutable derivative, Rodríguez-Rojas *et al*.[70,137] reported that the mutation frequencies of various fosfomycin combinations were typically greatly reduced compared to the individual antibiotics. These studies included combinations with tobramycin, ciprofloxacin and colistin (polymyxin E), all of which reduced the mutation frequencies in both strains to below the limit of detection ($<1\times 10^{-10}$ or $\leq 10^{-11}$). However, despite the promise for fosfomycin combinations to reduce the emergence of fosfomycin resistance suggested by these latter studies, our results in a time-kill model are less promising. In cases where the combinations led to extensive killing at 24 h, meaningful interpretation of the PAPs was not possible (*e.g.* tobramycin or ciprofloxacin against CR 1033 [non-MDR, susceptible to all antibiotics]). When significant regrowth was present, increases in Fos^R colonies in the PAPs with all combinations approximated those observed with equivalent monotherapy. However, as the emergence of resistance with combination therapy may be influenced by fluctuating as opposed to static antibiotic concentrations[291,292] it will be important to additionally assess the ability of these combinations to suppress fosfomycin-resistance in dynamic *in vitro* models; such investigations are currently underway in our laboratory.

While the mechanism(s) underpinning the observed enhanced pharmacodynamic effect with the various combinations are currently under investigation, one possible mechanism for enhanced bacterial killing involves increased uptake of one of the combination drugs by the other drug (so called mechanistic synergy[260,322]). Specifically for combinations containing polymyxin B or ciprofloxacin, enhanced bacterial killing may be due, at least in part, to increased penetration of fosfomycin facilitated by disruption of the bacterial outer membrane. Polymyxins initially bind to the lipopolysaccharide (LPS) of the outer membrane of Gram-negative organisms, ultimately causing membrane disruption and permeabilisation both to the polymyxin itself and other antibiotics.[323] Ciprofloxacin likewise has been shown to induce breakage of the outer membrane

of *P. aeruginosa*, an action independent of DNA breakage and impairment of the overall catalytic functions of DNA gyrase and topoisomerase IV, the primary targets of quinolones.[280,324,325] Enhanced penetration of fosfomycin across the outer membrane would theoretically be particularly useful against Fos^R isolates given a common mechanism of resistance to fosfomycin in *P. aeruginosa* is reduced uptake into the cell due to genetic mutation in the chromosomally encoded transport system GlpT (a G3P permease).[70,158,278] Interestingly, improvements in bacterial killing with the fosfomycin/polymyxin B combination were minimal in both Fos^R isolates (CW 11 and CW 30), whereas killing was substantially improved against the same isolates with the fosfomycin/ciprofloxacin combination (Tables 3.2 and 3.4). Recently, it has been suggested that accumulation of superoxides in the cell due to fosfomycin may be responsible for improved killing when combined with polymyxins.[259] In the reverse situation to that for polymyxin B and ciprofloxacin above, fosfomycin has been show to increase the active uptake of tobramycin into the cell, resulting in greater inhibition of protein synthesis and bacterial killing by the aminoglycoside.[282]

Conclusion

We have shown the combination of fosfomycin with polymyxin B or tobramycin at clinically relevant concentrations may substantially improve bacterial killing of Fos^S *P. aeruginosa*, while fosfomycin plus ciprofloxacin may substantially improve bacterial killing of Fos^R isolates. We have also shown for the first time that, in the absence of significant bacterial killing, none of these combinations could prevent an increase in Fos^R subpopulations in the presence of static drug concentrations. Further pre-clinical investigations using pharmacodynamic systems and animal models, coupled with mathematical modelling, are warranted to optimize each combination and reduce the emergence of resistance.

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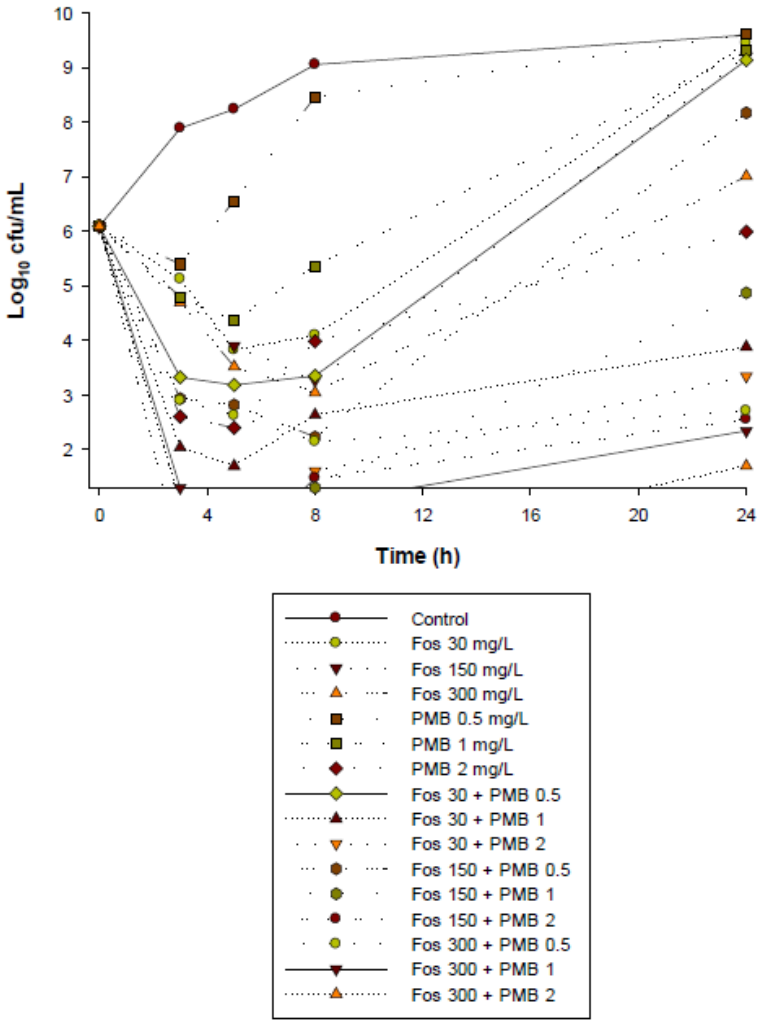
Transparency declarations

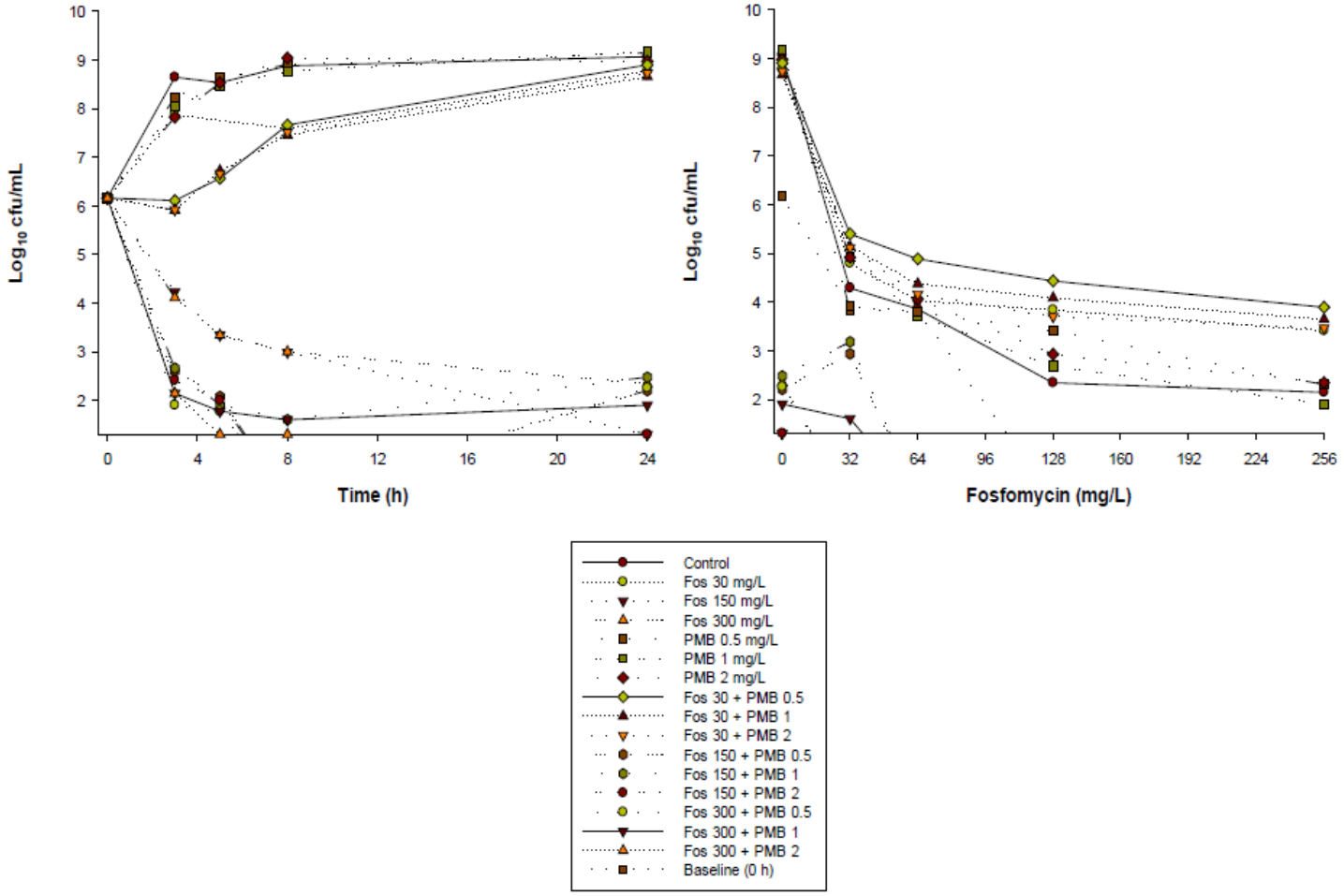
None to declare.

Supplemental material

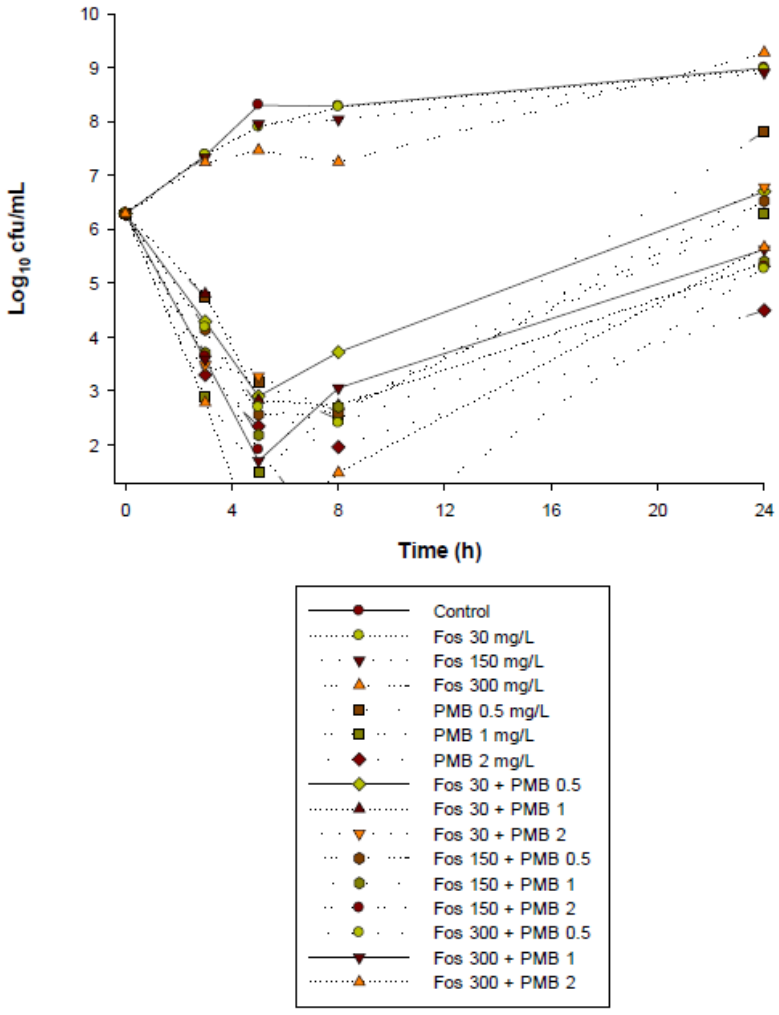
***P. aeruginosa* ATCC 27853** Time-kill curves for fosfomycin (Fos) and polymyxin B (PMB) alone and in combination.



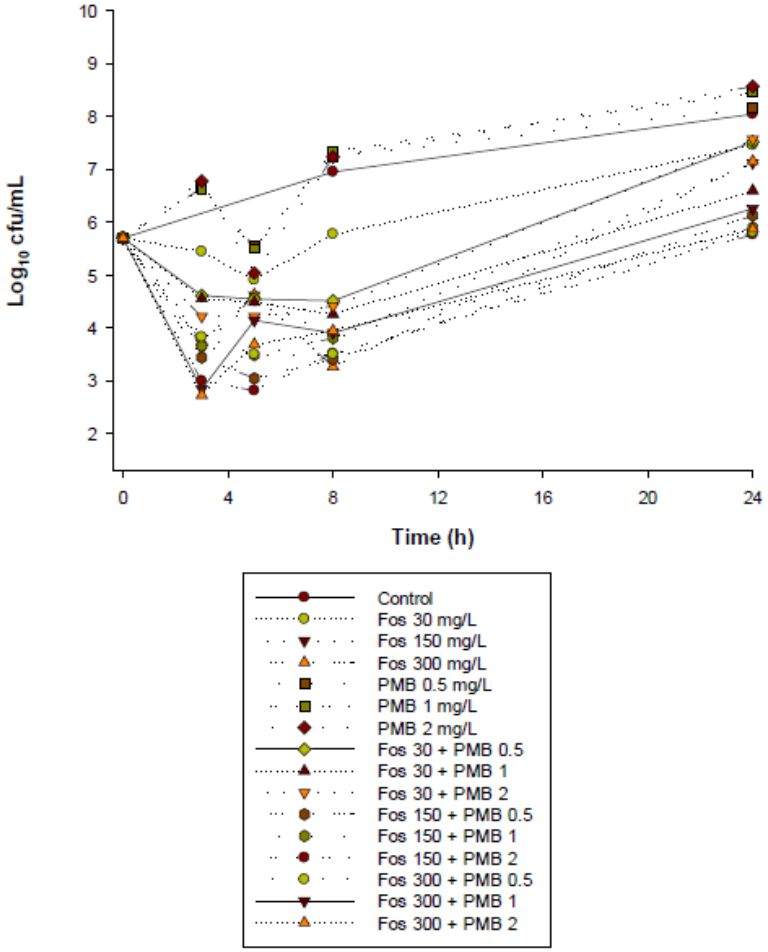
***P. aeruginosa* CW 2** Time-kill curves (left) and 0h (baseline) and 24h PAPs (right) for fosfomycin (Fos) and polymyxin B (PMB) alone and in combination.



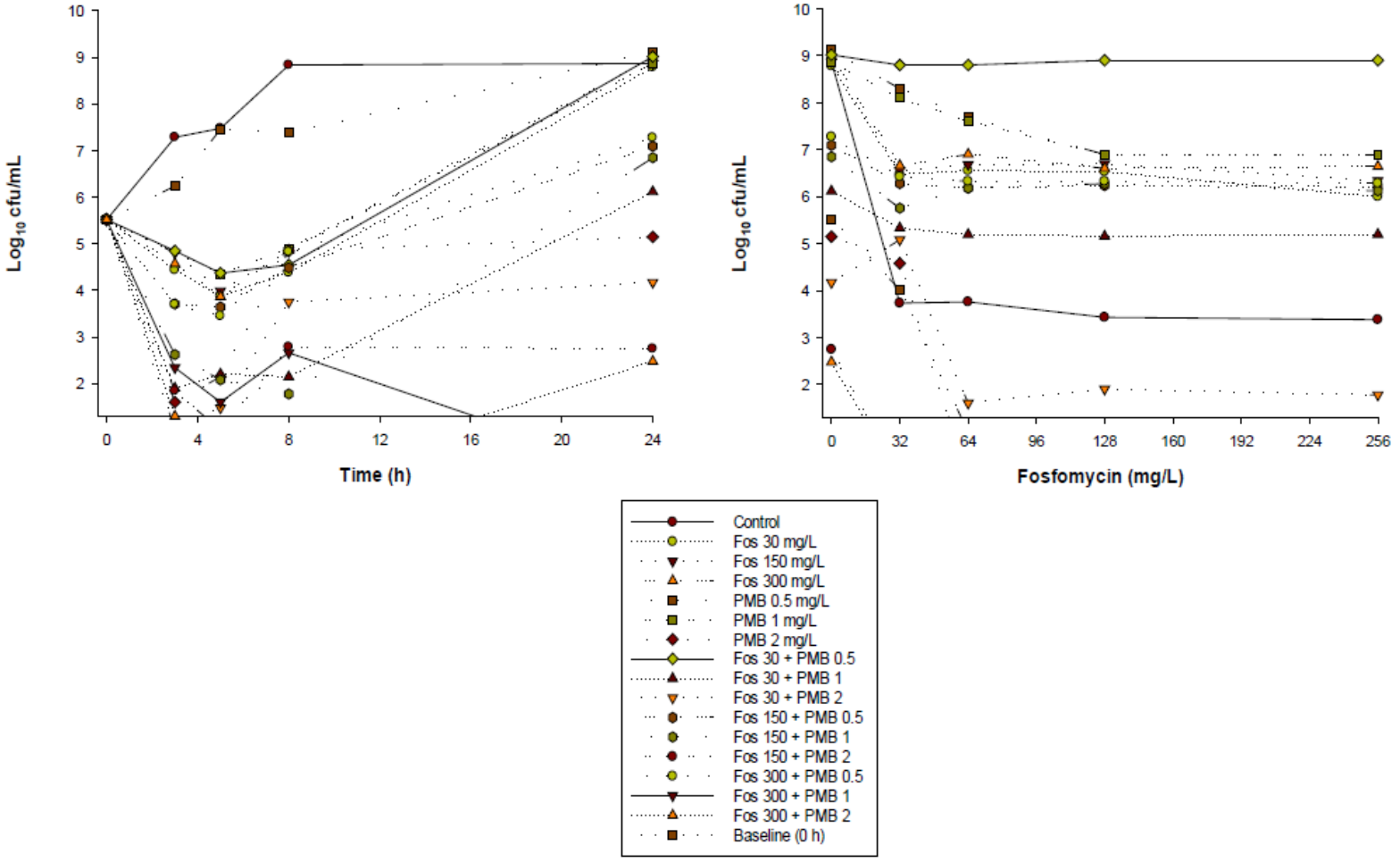
***P. aeruginosa* CW 11** Time-kill curves for fosfomycin (Fos) and polymyxin B (PMB) alone and in combination.



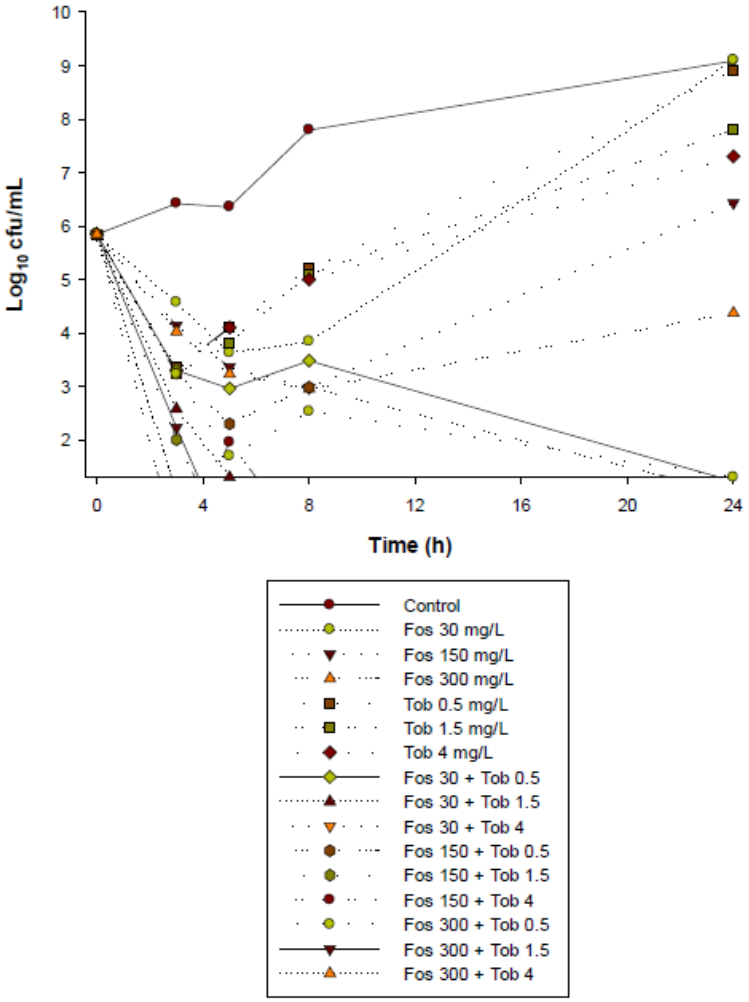
***P. aeruginosa* CW 30** Time-kill curves for fosfomycin (Fos) and polymyxin B (PMB) alone and in combination.



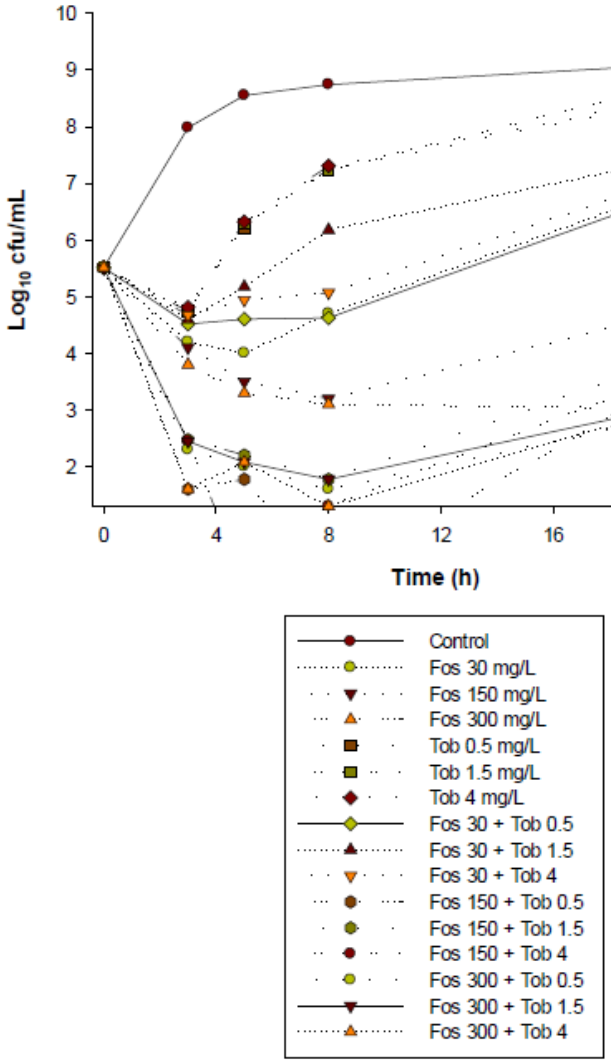
***P. aeruginosa* CR 1033** Time-kill curves (left) and 0h (baseline) and 24h PAPs (right) for fosfomycin (Fos) and polymyxin B (PMB) alone and in combination.



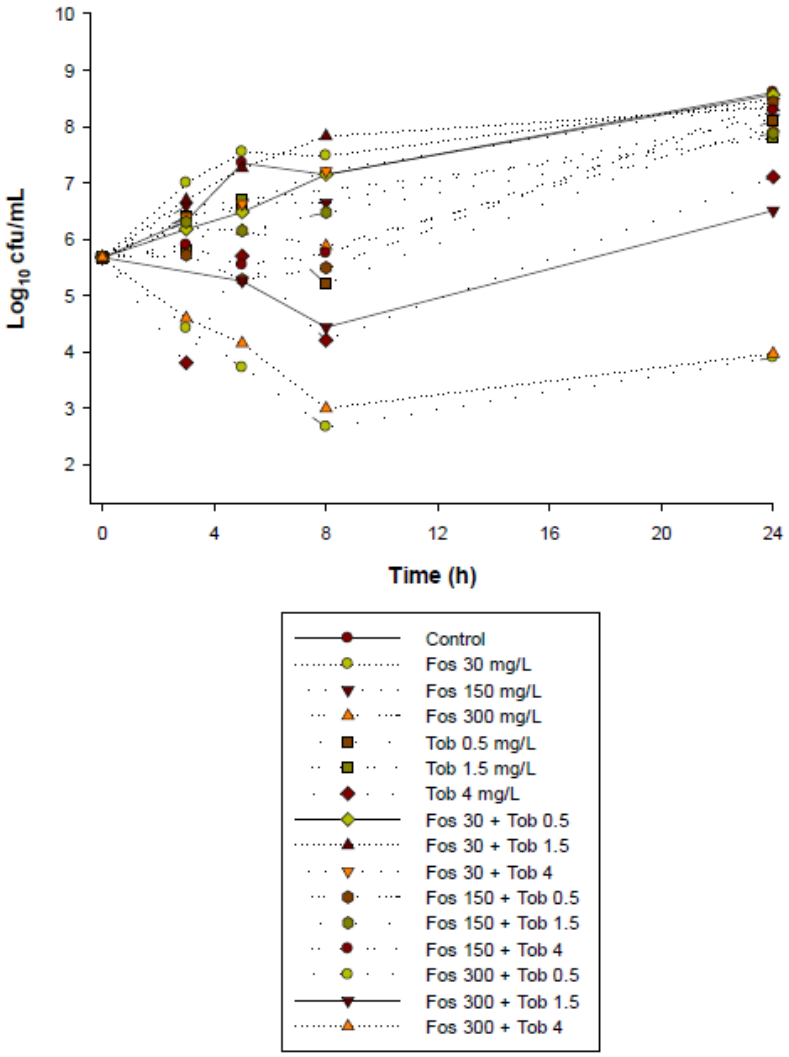
***P. aeruginosa* ATCC 27853** Time-kill curves for fosfomycin (Fos) and tobramycin (Tob) alone and in combination.



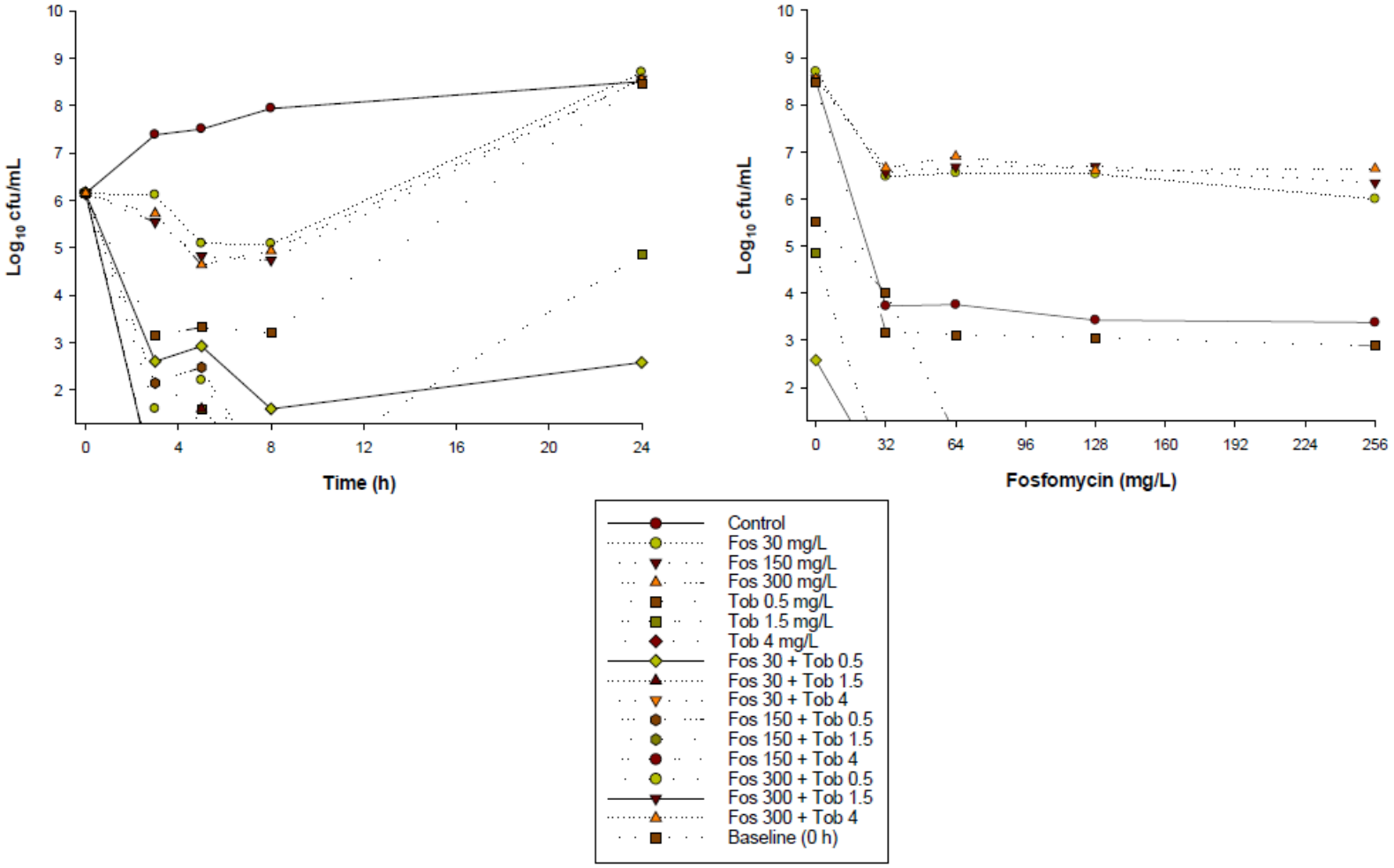
***P. aeruginosa* CW 2** Time-kill curves for fosfomycin (Fos) and tobramycin (Tob) alone and in combination.



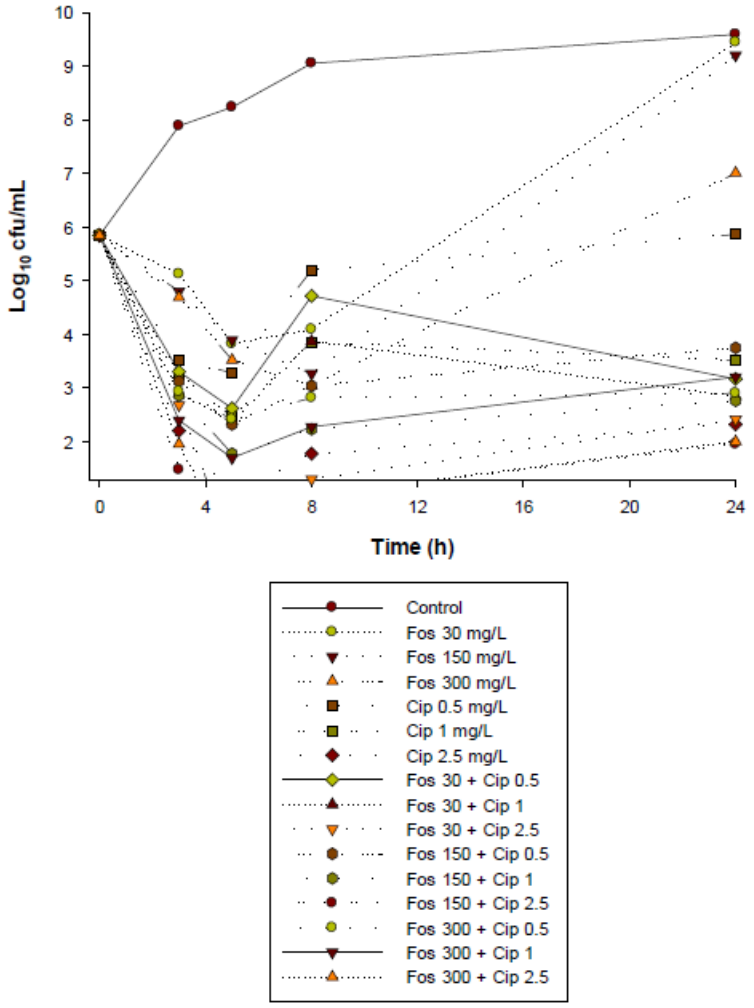
***P. aeruginosa* CW 11** Time-kill curves for fosfomycin (Fos) and tobramycin (Tob) alone and in combination.



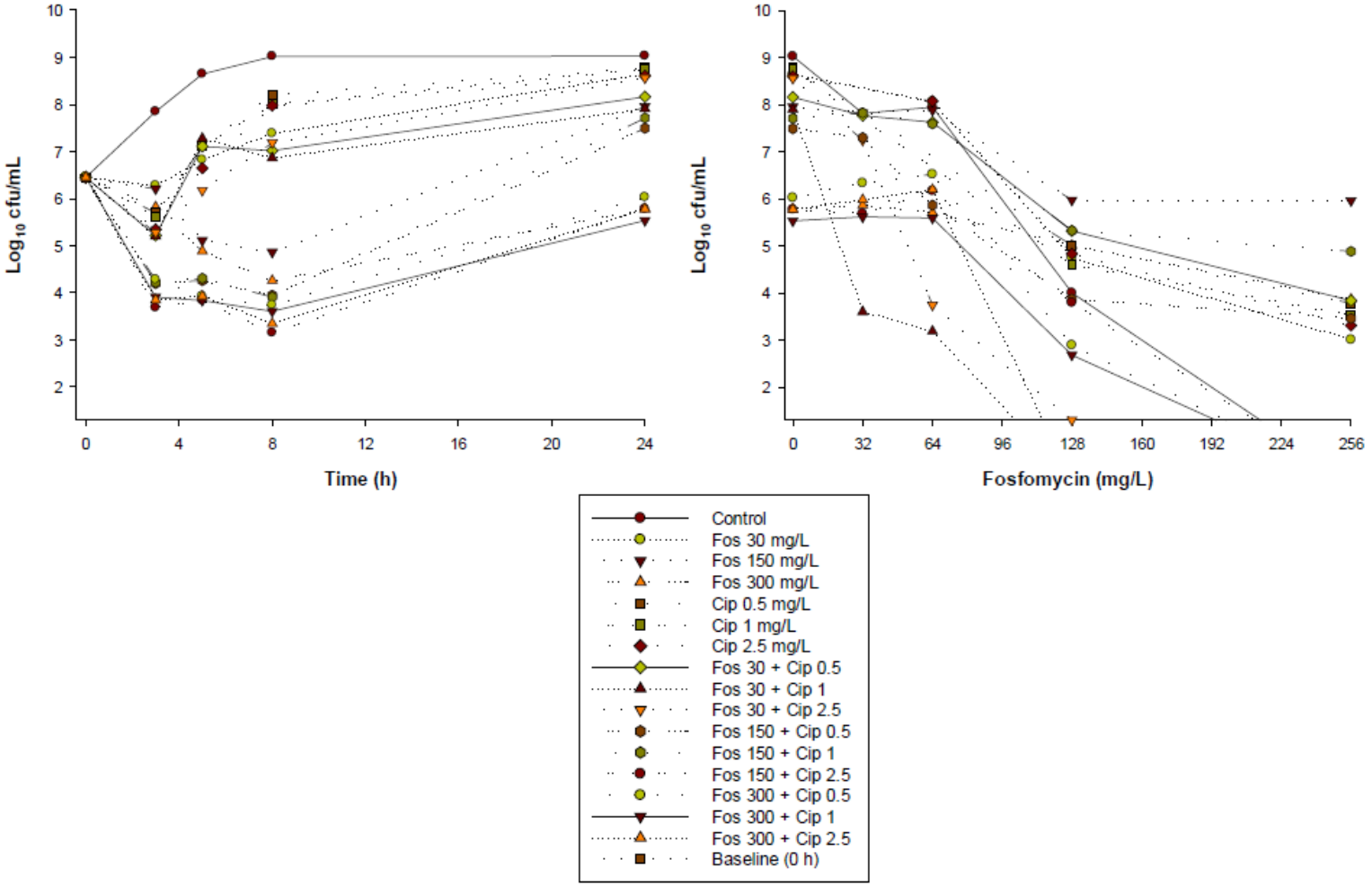
***P. aeruginosa* CR 1033** Time-kill curves (left) and 0h (baseline) and 24h PAPs (right) for fosfomycin (Fos) and tobramycin (Tob) alone and in combination.



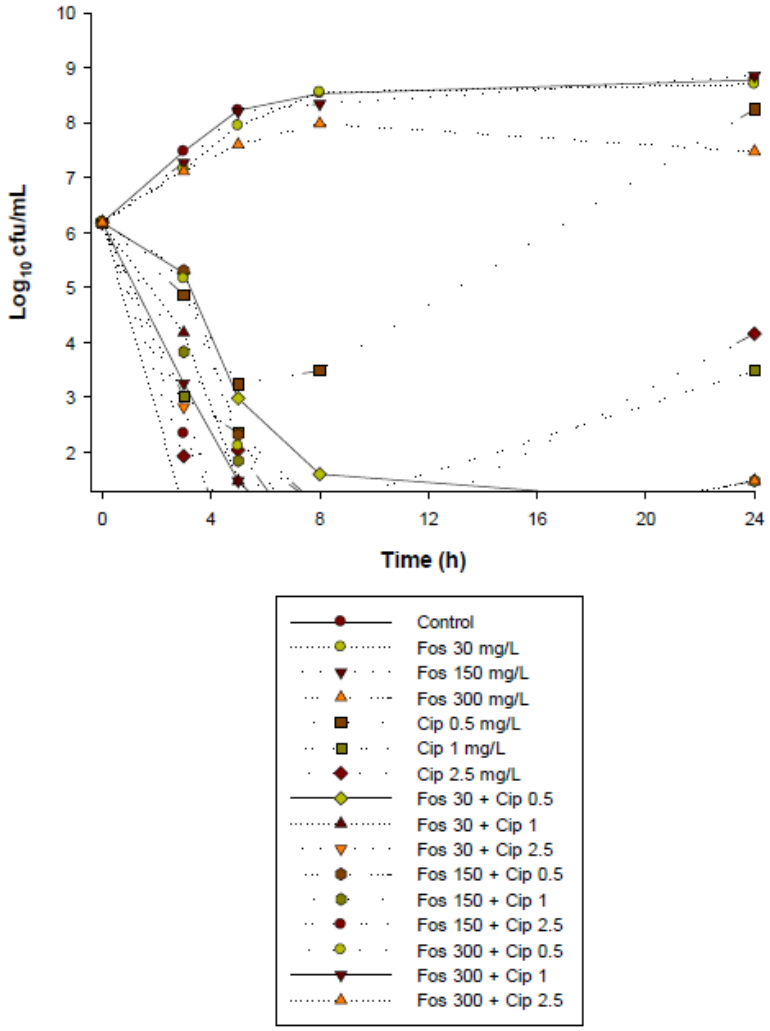
***P. aeruginosa* ATCC 27853** Time-kill curves for fosfomycin (Fos) and ciprofloxacin (Cip) alone and in combination.



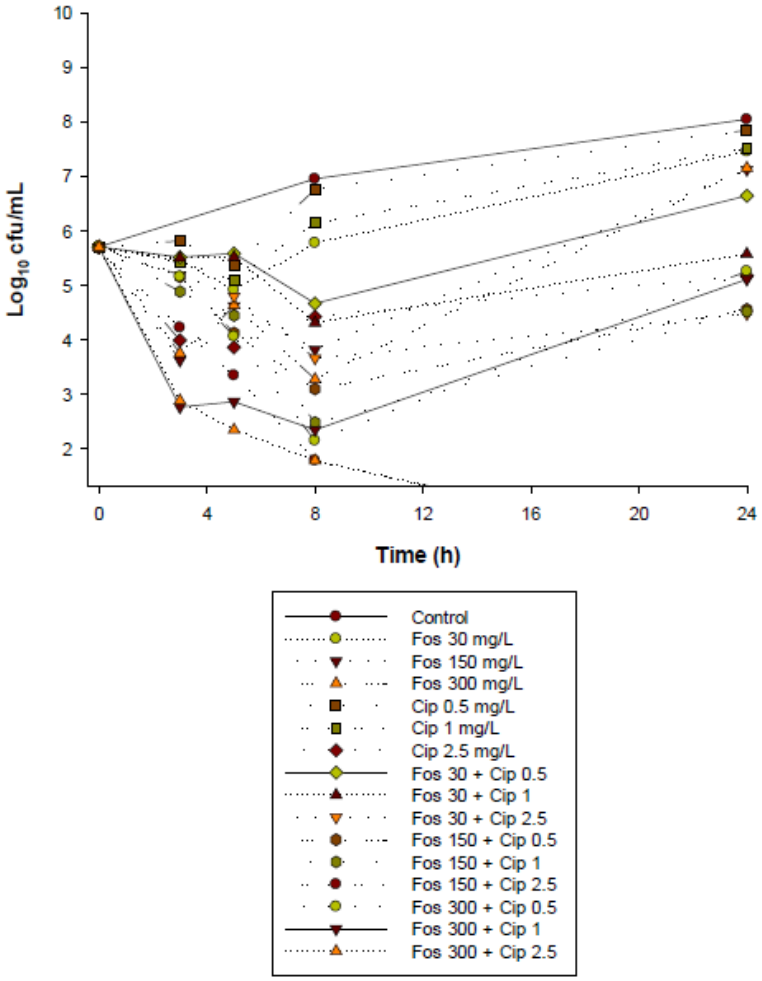
***P. aeruginosa* CW 2** Time-kill curves (left) and 0h (baseline) and 24h PAPs (right) for fosfomycin (Fos) and ciprofloxacin (Cip) alone and in combination.



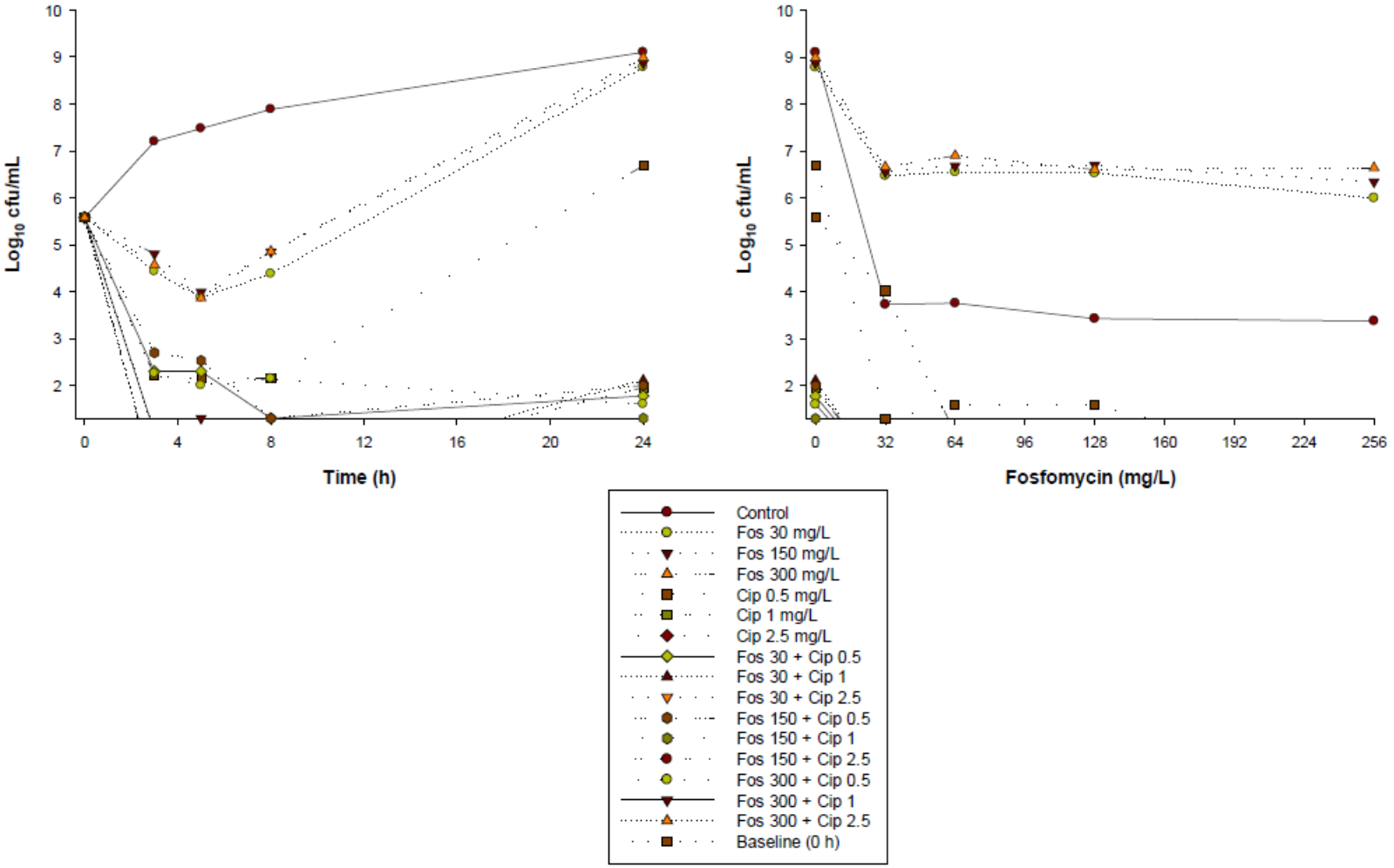
***P. aeruginosa* CW 11** Time-kill curves for fosfomycin (Fos) and ciprofloxacin (Cip) alone and in combination.



***P. aeruginosa* CW 30** Time-kill curves for fosfomycin (Fos) and ciprofloxacin (Cip) alone and in combination.



***P. aeruginosa* CR 1033** Time-kill curves (left) and 0h (baseline) and 24h PAPs (right) for fosfomycin (Fos) and ciprofloxacin (Cip) alone and in combination.



Chapter 4

Conclusions and future directions

The use of fosfomycin for treatment of systemic infections due to MDR bacteria is increasing due to rising resistance to other antibiotics and a marked decline in antibiotic discovery and development.[51,52,55,56,66-68] This concerning situation necessitates optimising therapy with currently available agents. Fosfomycin retains significant activity against a range of MDR Gram-positive[50,98] and Gram-negative bacteria[31,48,49,52-59,95-97,99-102] and is a promising agent, especially as part of combination therapy, for the treatment of various infections caused by such organisms.[70,110,138] Given this activity and a favourable adverse effect profile[32,55,60,66,78,139] there has been a recent increase in the use of fosfomycin for the treatment of systemic infections caused by MDR organisms, including those due to *P. aeruginosa*. However, fosfomycin was identified and developed prior to the introduction of modern drug development and approval procedures. Consequently, substantial gaps in our knowledge of the PK and PD properties of this antimicrobial currently exist. If known, these properties could be used to optimize the use of fosfomycin and improve patient care and outcomes by maximizing bacterial killing and minimizing the emergence of resistance.

Therefore, the aims of the two studies described in this thesis were to investigate the PD of fosfomycin alone, and in combination, against *P. aeruginosa* in order to facilitate optimisation of its clinical use against this pathogen. The first study (Chapter 2) systematically investigated for the first time the *in vitro* PD properties, namely MICs, PAPs, bacterial killing (including the effect of inoculum), and the PAE of fosfomycin against clinical isolates of *P. aeruginosa,* including MDR isolates. MICs ranged from 1 – >512 mg/L, with 61% of isolates considered fosfomycin-susceptible (MIC ≤64 mg/L). The MIC distributions for MDR and non-MDR isolates were similar. Baseline PAPs indicated heteroresistance in all isolates tested. Time-kill studies showed moderate, time-dependent killing at the low inoculum with regrowth at 24 h. All but the lowest concentration resulted in the complete replacement of

fosfomycin-susceptible colonies by -resistant colonies. Bacterial killing was virtually eliminated at the high inoculum. The PAE ranged from 0.3 – 5.5 h. This is currently the only study to examine the susceptibility of *P. aeruginosa* isolates to fosfomycin within an Australian context and to use multiple isolates to specifically examine whether killing of *P. aeruginosa* by fosfomycin is concentration- or time-dependent. It is also the only study to show the presence of fosfomycin heteroresistance in *P. aeruginosa* and an inoculum effect for fosfomycin against any organism. The data suggest fosfomycin monotherapy may be problematic for the treatment of infections caused by *P. aeruginosa*.

Resistance is known to develop rapidly when fosfomycin is used as monotherapy, particularly against *P. aeruginosa*. [52,70,110,137,253] As a consequence, combination regimens with a second antibiotic have been suggested to overcome this potential problem.[26,70,253] However, there is limited information, both pre-clinical and clinical, regarding which combination(s) maximize bacterial killing and minimize the emergence of fosfomycin resistance. Therefore, in Chapter 3 studies were undertaken against *P. aeruginosa* to systematically investigate bacterial killing and emergence of resistance with fosfomycin in combination with another antibiotic. As far as we are aware, this is the first study to examine the emergence of fosfomycin resistance with combination therapy over time against any organism.

The effects of fosfomycin-containing combinations (fosfomycin with one of tobramycin, polymyxin B or ciprofloxacin), as well as the effect of each antibiotic alone, were investigated using a static time-kill model. To investigate the emergence of fosfomycin resistance over time, PAPs were included at baseline and following 24 h of treatment. Against susceptible isolates, monotherapy produced varying degrees of initial killing followed by rapid regrowth. Fosfomycin plus polymyxin B or tobramycin produced greater initial killing (up to ~4-$\log_{10}$ cfu/mL) with many concentrations compared to monotherapy against fosfomycin-

susceptible isolates. Substantial improvements in killing were absent against fosfomycin-resistant isolates. For fosfomycin-ciprofloxacin combinations, improvements in bacterial killing were observed against fosfomycin-resistant isolates, while improvements in killing were largely absent against fosfomycin-susceptible isolates. However, no combination suppressed emergence of fosfomycin resistance. Investigations of fosfomycin combinations in *in vitro* PK/PD models, where the PK of fosfomycin in patients can be mimicked, represents an important future opportunity for research. The importance of such testing has been shown in the redevelopment of another old antibiotic, colistin, where a combination used at static concentrations failed to reduce the emergence of colistin resistance, whereas a similar combination in a dynamic (PK/PD) model resulted in a dramatic reduction of colistin-resistant subpopulations. Additionally, animal infection models and clinical studies are also warranted to optimize fosfomycin combinations targeting isolates resistant to all antibiotics, including fosfomycin.

Although the mechanism(s) underpinning the observed enhanced PD effect with the various combinations in Chapter 3 were not directly investigated as that was beyond the scope of this study, one possible mechanism for enhanced bacterial killing involves increased uptake of one of the combination drugs by the other drug (mechanistic synergy[260,322]). Alternatively, or in addition to mechanistic synergy, one drug may have killed the subpopulation(s) resistant to the other drug, and *vice versa* (subpopulation synergy[322]). The elucidation of the mechanism(s) underpinning the enhanced PD activity observed with the fosfomycin combinations investigated in Chapter 3 represents an opportunity for future investigations. Mechanism-based mathematical modelling will play an important role in predicting potential mechanisms and in proposing the most likely mechanisms.

Further to the additional studies outlined above, extending research in other areas will be beneficial to support the work presented in this thesis. The PK/PD index (i.e., the area under the unbound concentration-time curve to MIC ratio [$fAUC/MIC$], the unbound maximal concentration to MIC ratio [fC_{max}/MIC], or the cumulative percentage of a 24-h period that unbound concentrations exceed the MIC [$fT_{>MIC}$]) associated with maximal bacterial killing and minimization of resistance development in *P. aeruginosa*, as well as the magnitude of this index required to achieve various levels of bacterial killing, is required to further our understanding of fosfomycin PK and PD. The PK/PD index associated with maximal bacterial killing and minimal resistance can be determined through dose-fractionation studies performed via a dynamic (PK/PD) time-kill model or in animal models, with the inclusion of PAPs to monitor resistance rates. Once the PK/PD index is established, mathematical modelling should be performed with use of a population PK model to determine the probability of target attainment and expectation.[326,327] This work would provide further essential information for the optimization of fosfomycin therapy in patients with infections due to *P. aeruginosa,* and could also contribute towards the establishment of MIC breakpoints for fosfomycin against *P. aeruginosa*. These studies are currently underway in our research group.

In summary, this thesis has established a range of PD properties for fosfomycin use against *P. aeruginosa*. It has shown for the first time in *P. aeruginosa* the presence of fosfomycin heteroresistance and that the antimicrobial activity of fosfomycin is time-dependent. The first systematic investigation into fosfomycin combination therapy was undertaken, including the first examination of the emergence of fosfomycin resistance with combination therapy. The studies reported here have increased our understanding of fosfomycin PD, both in mono- and combination-therapy, and will assist in the design of optimal fosfomycin dosing regimens that will maximize bacterial killing and minimize the emergence

of fosfomycin resistance. Ultimately, I hope these findings will assist in achieving positive clinical outcomes in patients requiring this last-line antibiotic.

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